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13. ABSTRACT (Maximum 200 Words)

Type 1 diabetes is considered an autoimmune disease characterized by the presence of inflammatory cells in the islets of Langerhans. These cells are T lymphocytes, considered responsible for the destruction of the insulin producing beta-cells present in the islets. When the majority of the beta cells are dead, the disease presents, frequently with an abrupt and clinically serious onset. Individuals are considered at high risk to develop the disease, based on their genetic susceptibility (as determined by the presence of susceptibility alleles at various HLA loci) and on the presence in the serum of autoantibodies directed against islet specific autoantigens (e.g., GAD65, IA-2, and insulin). The aim of this program is to determine whom among the Army personnel is at high risk to develop the disease in order to prevent the unexpected onset of the disease that may be associated with tragic consequences, and to initiate an educational program aimed at reducing practical and psychological hurdles. Furthermore, different individuals develop disease complications (i.e., retinopathy, nephropathy, neuropathy) at different timepoints after the onset. The susceptibility to complications could also be genetic. The human genome will be scanned systematically to characterize these susceptibility genes. Proteomic analysis will be performed in tandem to confirm the genetic associations.

14. SUBJECT TERMS
Type 1 diabetes, HLA alleles, autoantibodies, T cells, DNA pyrosequencing, proteomic analysis

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INTRODUCTION TO THE OVERALL PROJECT

The research hypothesis on which our project is based is that there is a genetic contribution associated with increased risk for developing diabetic complications, specifically diabetic-nephropathy. That identifying SNP markers linked to this phenotype will enable improved prediction ultimately leading to advances in the prevention of this severe life threatening disease. The final aim of the entire project will be to provide a means to genetically test military personnel and families for their risk for developing diabetes and its complications. Before collecting samples from military personnel, we set out to demonstrate proof of principle by recruiting diabetics and their family members, who were visiting our Diabetes Clinic and the satellite clinic in Johnstown, and analyzing their specimens. Aim A.1 of the project is to build a prototype Research Sample Repository to receive, catalog, and store DNA, serum and urine samples from diabetics and their relatives to allow efficient HLA typing and mapping of genes promoting diabetes-associated complications, with particular emphasis on diabetic nephropathy initially, and then on retinopathy and finally on neuropathy, to compound them in an efficient predictive protocol for type 1 diabetes. Sub-Aim A.1.1 uses index cases to ascertain, recruit and prepare DNA for all appropriate members of families in which diabetic end-stage renal disease has occurred. Sub-Aim A.1.2 is to determine whether diabetic-nephropathy is linked to any of the proposed candidate genes. Aim A.2 of the research is to use proteomic analysis of specimens from complications affected patients and, by comparing them with diabetic patients without complications, to identify possible biomarkers for evaluating likelihood and/or severity of an individual's risk for developing diabetic complications and eventually propose possible lines of preventive treatments. All the results obtained in our genetic screening are duplicated blindly in Philadelphia at the University of Pennsylvania by Dr. Richard Spielman, so to have a necessary quality control for them. At the moment, the results obtained are practically overlapping . This is Aim A.3. Aim B is the progress report of the educational component of our program: the "Genetic Information for Testing Diabetes (GIFT-D)".

A. DIABETIC COMPLICATIONS TYPING

A.1 Diabetic Nephropathy Susceptibility Genes

Specific Aim 1--Genetic Research (A.1.1): Single nucleotide polymorphisms (SNPs) are being analyzed for linkage to the diabetic-nephropathy phenotype. The patterns of allelic inheritance from parents to affected offspring are being evaluated by transmission/disequilibrium testing (TDT) of family trios (father, mother, and affected offspring) for genetic linkage of specific alleles to the phenotype (Ewens and Spielman, 1995). Multiple sources of diabetic-nephropathy families have been collected. These include cases obtained from the Children's Hospital of Pittsburgh collection of extended families (Figure 1. *NB: The figures are shown in Appendix 1*), family trios being actively recruited from the University of Pittsburgh School of Medicine Transplantation Institute

(Table 1), and the Juvenile Diabetes Research Foundation collection of diabetic family trios (Morel et al., 1988). Additional families will be recruited at the University of Hawaii and at Walter Reed Hospital in Washington, DC, pending their respective IRB approvals. Together these sources of genomic DNA samples provide more than 800 family trios of parents and patient, and extended families, for genetic analysis (Table 2). Based on the 40% prevalence of diabetic-nephropathy it is anticipated that our cohort will provide at least 300 families with the diabetic-nephropathy complication.

(A.1.2) Candidate loci used for TDT analysis were chosen based on the results of previous studies associating various regions of the human genome to the phenotype (Table 3). For example, the COL4A1 locus on chromosome 13 encodes an extracellular matrix protein and has been associated with increased fibrosis of the kidney in individuals suffering from nephropathy (Ziyadeh et al., 2000). At this point in our study, the COL4A1 locus has been studied for 58 genetic transmissions from parent to affected offspring. TDT statistical analysis supports the hypothesis for significant genetic linkage of COL4A1 to the nephropathy phenotype, exhibiting a p-value < 0.005. Other loci, such as Protein Kinase C, Beta 1 (PRKCB1) have been examined among 45 transmissions from parent to patient and, presenting a p-value of 0.05, also indicate a statistically significant linkage to the phenotype. The enzymatic action of Protein Kinase C has been associated with increased activity in patients exhibiting nephropathy disease (Araki et al., 2003). The overall genotyping strategy is to complete the analysis of these candidate loci using the parents and patient trios from the diabetic families that have been collected. Moreover, these sources consisting of family DNAs as well as patient health history (e.g., type of diabetes, age of diabetic onset, and duration of diabetes prior to onset of nephropathy) may also be useful for stratifying the cohort in order to better evaluate the significance of linkage of a particular inherited allele with the severity of the complication.

In order to enable the genotyping process, Pyrosequence-Based Typing (PSBT) assays have been designed for SNPs associated with the loci of interest. As of the end of Fiscal Year 2, PSBT assays for 30 of the genetic loci have been validated for use with the patient samples collected for this project (Table 4). In order to continue the successful design of PSBT assays for the complete set of candidate loci, computer software has been written to provide primers for PCR and pyrosequencing that are optimized for the diabetic-nephropathy project (Figure 2 in Appendix 1). Available at URL: http://biodev.hgen.pitt.edu/sop3 the software is a web-based program capable of designing assays for genotyping large numbers of SNPs (Alexander et al., 2004). The program accepts as input either gene locus names or SNP identification numbers. The output is a list of oligonucleotide primers used during PSBT to evaluate the inheritance patterns of SNP markers, thus allowing the collection of genetic data for analysis of their linkage to the disease phenotype (Figure 3 in Appendix 1).

The PSBT method is suited for use in the evaluation of the linkage between genomic markers and disease phenotype (Ringquist et al., 2004, 2002). The instrumentation provides genomic sequence analysis from 96 samples within 15 minutes. The 2 Pyrosequencer HS-96 instruments dedicated to this study are equipped with 10-tray robotic stacking arms that allow the genotyping of as many as 1920

samples in a batch, and roughly 1 batch can be prepared, run, and analyzed in a day. Validation of PSBT assays for detection of SNP transmissions has been accomplished through the use of gradient thermocycler instruments. The gradient thermocyclers have been used to aid in the optimization of PCR conditions, optimization of the PCR step increases the yield of sequencing template for PSBT (Figure 4 in Appendix 1). PCR primers are considered as validated once a robustly amplified product of the correct molecular weight is established. Confirmation of successful PCR is followed by analysis of the results of the pyrosequencing reaction, which are validated if they produce computer software readable sequencing information using the PSBT genotyping software provided by the instrument manufacturer, Biotage Inc. Successful primer sets are then used for genotyping analysis of family trios.

A strategy has been developed for reducing the likelihood of sample cross contamination that is commonly associated with repeated amplification of the same genomic region, as will be necessary during the diabetic-nephropathy genotyping project (Figure 5 in Appendix 1). Use of dUTP during PCR provides a method of containing this anticipated problem (Ritzler et al., 1999). The resulting dU-PCR products provide high quality PSBT data during pyrosequencing and are sensitive to degradation after exposure to the enzyme Uracyl-ADN Glycosylase, thus insuring removal from the laboratory once the genomic data has been acquired. The standard operating protocol of the genotyping laboratory will be to treat new samples of genomic DNA with the Uracyl-AND Glycosylase enzyme prior to PCR amplification of new genomic DNA, "cleaning" these samples of accidental contamination with the products of the previous days' sequencing templates.

PSBT data and TDT linkage analysis has been performed using allelic transmission data obtained from genotyping as many as 76 transmissions from families available for this study. An example of the PSBT data obtained for SNPs located within the LPL and PRKCB1 loci are shown in Figure 6. A summary of the transmission data indicates possibly significant linkage of 11 loci with the phenotype (Table 5). Among these loci, the most significant linkages observed, p-values < 0.005, encode the proteins B-cell Lymphoma Protein 2, Collagen Type IV Alpha 1 Chain, and Lipoprotein Lipase indicated by the locus symbols BCL2, COL4A1, and LPL, respectively. These genomic regions have also been linked to a variety of complications-related ailments. For example, abnormalities in the BCL2 encoded protein may affect kidney morphogenesis by negatively influencing inductive interactions between epithelium and mesenchyme, important processes during kidney organogenesis (Nakayama et al., 1994).

A.2 Diabetic Nephropathy Proteomic Analysis

Specific Aim 2--Proteomic Research: The purpose of the proteomic research effort is to examine protein samples collected from patients and control populations in order to establish methods for identifying protein markers whose appearance/disappearance correlate with disease. These samples provide the basis for developing biological markers that, along with the genetic markers obtained as a result of Specific Aim 1, can be used in evaluating an individual's risk for developing diabetic complications. The

proteomic research has focused, first, on evaluating the changes in protein expression from healthy human pancreatic islet tissue, obtained as part of our collaboration with the University of Pittsburgh School of Medicine human organ donor program. These analyses seek to allow the determination of the possible association of specific proteins with successful use of this tissue during islet transplantation as a treatment for diabetes and diabetic complications. Pancreatic islet transplantation has been correlated with improved patient well-being due to reduced prevalence and severity of diabetic complications (Fiorina et al., 2003). The goal of this phase of the research being to correlate individual proteins isolated from human pancreatic islet tissue with successful transplant outcome and to evaluate the usefulness of these proteins as markers for predicting, improving, and monitoring the likelihood of successful islet transplantation in the treatment of diabetes and its complications.

The first aim of the proteomic strategy has been realized in that it has established the possibility of comparing the islet proteome from samples collected from different human organ donors, a necessary step for evaluating biomarkers for transplantation. Data collected from 5 pancreas organ donors has indicated that proteins visualized by 2D gel electrophoresis are, in fact, constant for all but one set of observed proteins (Figures 7 and 8 in Appendix 1). The 3 variable proteins were, however, not of human origin and accumulate in the sample as a result of the requirements of the islet isolation protocol. That is, purification of healthy islet tissue suitable for transplantation requires the addition of carrier proteins to the preparation. In the samples studied thus far bovine serum albumin has been included as a carrier protein to improve the yield of islet tissue capable of robust glucose-stimulated insulin release. Analysis of the variable proteins by mass spectroscopy confirmed their identity as isoforms of bovine albumin (Figure 9 in Appendix 1) while analysis of the remaining proteins, a class of proteins whose abundance are constant among our organ donor population, indicated that they are of human origin.

As noted above, analysis of pancreatic islet tissue isolated from genetically diverse individuals has provided similar proteomic signals, indicated as high as a 98% degree of similarity (Figure 10 in Appendix 1). These results have, in turn, allowed experiments to proceed that test the response of the islet proteome to exposure to different isolation/culturing conditions as well as isolation of functional protein compartments, such as insulin granule associated proteins. For example, comparison of protein populations associated with total islet and insulin granule fractions indicate a nearly quantitative difference in the molecular species present, confirming the success of the insulin granule isolation protocol (Figure 11 in Appendix 1). Continuing work on these projects will exploit mass spectroscopy to provide identification of protein species in order to evaluate the role of specific proteins in islet cell biology with the goal of improving their use as a transplant resource for treating diabetes and its complications.

KEY RESEARCH ACCOMPLISHMENTS:

Research Accomplishments Emanating from the Research Project

- Greater than 800 trios (father, mother, and patient probands) have been obtained

to allow identification of genetic markers linked to diabetic complications, including diabetic-nephropathy.

- Development, testing, and publication of software applications for design of genotyping assays.
- 30 validated PCR and SNP assays available for genetic typing.
- Allele transmission data on over 1,400 events from 11 different genetic loci.
- Development and testing of a prototype Lab Information Management System (LIMS) for use in analysis of genotyping data and sample/inventory management.

REPORTABLE OUTCOMES:

Manuscripts: While a total of 28 peer-reviewed publications have been submitted by the P.I's group in the reporting period, there have been 7 relevant to this program.

1. Friday RP, Profozich J, Pietropaolo S, **Trucco M**, Pietropaolo M: Alternative core promoters regulate tissue-specific transcription from the autoimmune diabetes-related *ICA1* (ICA69) gene locus. **J Biol Chem** 278:853, 2003.
2. Bottino R, Lemarchand P, **Trucco M**, Giannoukakis N: Gene and cell-based therapeutics for type 1 diabetes mellitus. **Gene Therapy** 10:875, 2003.
3. Giannoukakis N,**Trucco M**: Gene therapy technology applied to disorders of glucose metabolism: promise, achievements and prospects. **BioTechniques** 35:122, 2003.
4. Dorman JS, Charron-Prochownik D, Siminerio L, Ryan C, Poole C, Becker D, **Trucco M**: Need for genetic education for type 1 diabetes (letter). **Arch Pediatr Adolesc Med** 157:935, 2003.
5. Ringquist S, Alexander AM, Styche A, Pecoraro C, Rudert WA, **Trucco M**: HLA class II DRB high resolution genotyping by pyrosequencing: comparison of group specific PCR and pyrosequencing primers. **Human Immunol** 65:163, 2004.
6. Barinas-Mitchell E, Pietropaolo S, Zhang YJ, Henderson T, **Trucco M** , Kuller LH, Pietropaolo M: Islet cell autoimmunity in a triethnic adult population of the Third National Health and Nutrition Examination Surveys (NHANES) III. **Diabetes** 53:1181, 2004.
7. Alexander AM, Pecoraro C, Styche A, Rudert WA, Benos PA, Ringquist S, **Trucco M**: SOP^3: A web-based tool for selection of oligonucelotide primers for single nucleotide polymorphism analysis. **BioTechniques**, In press, 2004.

Meeting Abstracts and Presentations

1. Ringquist S, Ge X, Zhang L, Styche L, Balamurugan AN, Bottino R, Rudert WA, Trucco M. Proteomic scanning for markers associated with successful islet isolation and maintenance. Keystone Symposia Diabetes Mellitus. 2004.
2. Pecoraro C, Styche A, Rudert WA, Benos PV, Ringquist S, Trucco M. SOP^3: A web based tool for selection of oligonucleotide primers for SNP analysis. University of Pittsburgh Science 2004 Symposia. 2004.

Patents and Licenses (applied for and/or issued)

- Copyrighted Material: SOP3 software for designing primers for PCR and pyrosequencing.

Degrees Obtained that are Supported by this Award

- N/A

Development of Cell Lines

- Lymphoblastoid cell lines from the Children's Hospital of Pittsburgh collection of diabetic families.

Tissue or Serum Repositories

- Repository of almost 2500 DNA samples from 827 families made up of diabetic trios, consisting of father, mother, and affected offspring.

Informatics and Databases

- Development, testing, and publication of software application for designing assays for genotyping of human DNA samples.
- Warehousing of databases of the human genome, human genetic polymorphisms, and human haplotype maps.

Animal Models

- N/A

Funding Applied for Based on Work Supported by this Award

- None

Employment or Research Opportunities Applied for and/or Received Based on this Award

- None

CONCLUSIONS:

Summary of the Importance and/or Implications of the Research

Healthcare costs associated with diabetes and diabetic complications, such as nephropathy, account for 10% of monies spent on healthcare. Early identification of individuals at risk for this chronic disease will aid in improved management, decreased severity, and reduced healthcare costs. Potential benefits of the project are: (1) improved forecasting of the genetic risk of developing diabetes and diabetic complications; and (2) the opportunity to apply preventative treatment focused on at-risk individuals.

Recommended Changes on Future Work

Challenges to successful completion of the project are as follows:

1. Obtaining the quantity of samples to be analyzed in order to provide statistically significant results.

We have arranged through our association with the Children's Hospital of Pittsburgh, the University of Pittsburgh School of Medicine Transplantation Institute, the University of Hawaii, and the Walter Reed Hospital in Washington, DC, and the Juvenile Diabetes Research Foundation to receive genomic DNA samples from at least 300 family trios (father, mother, and diabetic offspring) in order to evaluate a sufficiently large number of allelic transmissions for statistically significant genotyping analysis. These samples consist of genomic DNA as well as health records to allow evaluation of the presence of diabetic complications. The materials will become available at the beginning of 2005, but the collection will continue throughout the entire year.

2. The need to validate alternative methods for monitoring the accuracy of the genotyping data.

During the current year of the project we will introduce alternative methods for genotyping our family trios, the goal being to confirm the accuracy of the primary genotyping data by re-genotyping 4% of the available family trios. This will be preformed using a human polymorphism microarray manufactured by Illumina Corporation and operated by the University of Pittsburgh School of Medicine Genomic Core Laboratory. The throughput of this system will allow, in a single run, genotyping of 5,861 loci from 96 samples (or 32 family trios), providing confirmation of the pyrosequence-based genotyping protocol as well as extending our association and linkage analysis to other genomic loci.

3. Continued development and testing of software for data analysis as well as inventory management.

Development of laboratory data management software and custom databases for the warehousing of genomic information will be pursued on an ad hoc basis. We currently have a prototype LIMS application for sample/inventory tracking and will expand its functions to include analysis of genotyping data.

Military Relevance of the Research Project:

- Diabetes affects 16 million Americans (greater than 5% of the population), and 800,000 new cases annually.
- Diabetes occurs in men, women, children and the elderly. African, Hispanic, Native and Asian Americans are particularly susceptible to its most severe complications.
- Diabetic kidney disease accounts for 42% of new cases of end-stage renal disease, with over 100,000 cases per year at an average cost of $55,000 per patient annually.

- Economic impact of diabetes is over $100 billion annually accounting for more than $1 in every $10 healthcare dollars and $1 of every $4 Medicare dollars spent.
- As the military is a reflection of the US population, improved prediction of risk for developing diabetes and diabetic complications amongst active duty members of the military, their families and retired military personnel will potentially allow focused preventative treatment of at-risk individuals, providing significant healthcare savings and improved patient well-being.

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Araki S, Ng DP, Krolewski B, Wyrwicz L, Rogus JJ, Canani L, Makita Y, Haneda M, Warram JH, Krolewski AS. Identification of a common risk haplotype for diabetic nephropathy at the protein kinase C-beta1 (PRKCB1) gene locus. J Am Soc Nephrol. 2003 14:2015-2024.

Ewens WJ and Spielman RS. The transmission/disequilibrium test: history, subdivision and admixture. Am. J. Hum. Genet. 1995 57:455-464.

Fiorina P, Folli F, Bertuzzi F, Maffi P, Finzi G, Venturini M, Socci C, Davalli A, Orsenigo E, Monti L, Falqui L, Uccella S, La Rosa S, Usellini L, Properzi G, Di Carlo V, Del Maschio A, Capella C, Secchi A. Long-term beneficial effect of islet transplantation on diabetic macro-/microangiopathy in type 1 diabetic kidney-transplanted patients. Diabetes Care 2003 26:1129-1136.

Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M. Aspartic acid at position 57 of the HLA-DQB chain protects against type I diabetes: a family study. Proc. Natl. Acad. Sci. USA 1988 85:8111-8115.

Nakayama, K.; Nakayama, K.; Negishi, I.; Kuida, K.; Sawa, H.; Loh, D. Y. Targeted disruption of Bcl-2-alpha-beta in mice: occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. Proc. Nat. Acad. Sci. USA 1994 91:3700-3704.

Ringquist S, Alexander AM, Styche A, Pecoraro C, Rudert WA, Trucco M. HLA class II DRB high resolution genotyping by pyrosequencing: comparison of group specific PCR and pyrosequencing primers. Hum Immunol. 2004 65:163-174.

Ringquist S, Alexander AM, Rudert WA, Styche A, Trucco M. Pyrosequence-based typing of alleles of the HLA-DQB1 gene. Biotechniques 2002 33:166-175.

Ritzler M, Perschil I, Altwegg M. Influence of residual uracil-DNA glycosylase activity on the electrophoretic migration of dUTP-containing PCR products. J Microbiol Methods 1999 35:73-76.

Ziyadeh FN, Hoffman BB, Han DC, Iglesias-de la Cruz MC, Hong SW, Isono M, Chen S, McGowan TA, Sharma K. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. Proc. Nat. Acad. Sci. USA 2000 97:8015-8020.

Table 1. University of Pittsburgh Transplant Institute Diabetic-Nephropathy Pedigrees.

ID	Relationship	Age	DM	Duration	DN	ID	Relationship	Age	DM	Duration	DN
7001-01	Father	67 yrs				7012-01	Father		Deceased		
7001-02	Mother	64				7012-02	Mother		64 yrs		
7001-03		38	DM		Yes	7012-03		44	T1DM	20 yrs	Yes
7003-01	Father	81				7013-01	Father	Deceased			
7003-02	Mother	73				7013-02	Mother	71			
7003-03		34	T1DM	34 yrs	Yes	7013-03		44	T1DM	26	Yes
7004-01	Father	66				7014-01	Father	Deceased			
7004-02	Mother	65				7014-02	Mother	77			
7004-03		37	T1DM	33	Yes	7014-03		40	T2DM	40	Yes
7005-01	Father	Deceased				7016-01	Father	73			
7005-02	Mother	82				7016-02	Mother	68			
7005-03		52	T2DM	13	Yes	7016-03			T1DM	17	Yes
7007-01	Father	76				7017-01	Father	Deceased			
7007-02	Mother	71				7017-02	Mother	49			
7007-03		39	T2DM	15	Yes	7017-03		31	T1DM	18	Yes
7008-01	Father	55				7018-01	Father	82			
7008-02	Mother	51				7018-02	Mother	78			
7008-03		31	T1DM	24	Yes	7018-03		42	T1DM	27	Yes
						7018-04	Brother	40	T1DM	32	No
7009-01	Father	67				7019-01	Father	75			
7009-02	Mother	64				7019-02	Mother	74			
7009-03		38	T1DM	24	Yes	7019-03		49	T1DM	37	Yes
7010-01	Father	72				7020-01	Father	70			
7010-02	Mother	70				7020-02	Mother	Deceased			
7010-03		41	T2DM	16	Yes	7020-03		43	T1DM	27	Yes
7011-01	Father	Deceased									
7011-02	Mother	63									
7011-03		40	DM	32	Yes						

Abbreviations are: Diabetes Mellitus, DM; Diabetic-Nephropathy, DN; Type 1 Diabetes Mellitus, T1DM; Type 2 Diabetes Mellitus, T2DM.

Table 2. Summary of Diabetic-Nephropathy Family Trios.

Sources of Family Trio Genomic DNA

Thomas E Starzl Transplantation Institute
(IRB/HSRRB approval received; annual HSRRB approval pending)
Children's Hospital of Pittsburgh
(sample repository, no patient identifiers)
Juvenile Diabetes Research Foundation
(sample repository, no patient identifiers)
University of Hawaii
(pending IRB/HSRRB approval)
Walter Reed Hospital, Washington, DC
(pending IRB/HSRRB submission)

827 Total Numbers of Diabetic Family Trios consisting of father, mother, and affected offspring.

Table 3. Candidate Genes for Diabetic-Nephropathy.

Extracellular Matrix	Enzymes	Cytokines and Growth Factors
Collagen4A1	Aldose Reductase	Fibroblast growth factor 2 (basic)
Collagen4A2	Angiotensin Converting Enzyme	Insulin-like growth factor 1
Collagen4A3	Cathepsin B	Insulin-like growth factor binding protein-1
Collagen4A4	Endothelin converting enzyme 1	Platelet-derived growth factor beta
Collagen4a6	Metalloproteinase-3	Transforming growth factor-beta1
Fibronectin 1	Protein kinase C, alpha	Transforming growth factor-beta2
Integrin, alpha 2	Protein kinase C, beta 1	Transforming growth factor-beta3
Integrin, alpha V	Renin	
Integrin, beta 1	Metalloproteinase 2	Transcription Factors
Laminin A4	Metalloproteinase 3	c-fos
Laminin B1		c-jun
Laminin B2	Receptors	c-myc
Nidogen	AGE receptor	
	Angiotensin-2 receptor 1A	Others
Hormones	Endothelin receptor A	Apolipoprotein-E
Atrial natriuretic factor	Endothelin receptor B	Glucose transporter-1
Adrenomedulin	Insulin-like growth factor 1 receptor	Na+/H+ antiporter
Angiotensin	Insulin-receptor related receptor	
Preproendothelin	PDGF receptor beta	
	Transforming growth factor-beta receptor II	
	Transforming growth factor-beta receptor III	

Table 4. 30 Validated Pyrosequencing Assays for TDT analysis.

Locus	SNP ID	Allele	Het	Function	Location	Chromosome
APOE	rs429358	T/C	0.25	nonsyn	50103781	19
BCL2	rs1481031	C/T	0.52	intron	59001054	18
BCL2	rs2062011	A/T		intron	59025083	18
COL4A1	rs679062	T/C		intron	108597350	13
COL4A2	rs4771663	C/G		intron	108677798	13
COL4A2	rs4773161	A/G		intron	108695195	13
COL4A2	rs4773175	C/G		intron	108744573	13
COL4A5	rs2143442	G/A		intron	106510088	X
COL4A5	rs2272946	T/G		nonsyn	106606310	X
COL4A5	rs3747408	G/A		syn	106621933	X
COL4A5	rs4308887	A/G		intron	106612291	X
COL4A5	rs5929099	C/A		intron	106478705	X
COL4A5	rs5929138	A/T		intron	106631208	X
ITGA2	rs2056402	A/T	0.20	intron	52379511	5
ITGA2	rs26679	C/G	0.50	acc null	52300060	5
ITGA2	rs27890	A/G	0.20	intron	52341933	5
ITGA2	rs3212594	A/G	0.19	syn	52386829	5
ITGB1	rs1316757	A/C	0.42	intron	33201203	10
ITGB1	rs2256455	G/T		intron	33223014	10
LAMC1	rs2296292	A/C	0.47	syn	180326138	1
LAMC1	rs4652775	A/T		intron	180301206	1
LAMC1	rs7410919	T/C		nonsyn	180333928	1
LAMC1	rs7556132	A/G		nonsyn	180325136	1
LPL	rs13702	T/C		utr	19834765	8
LPL	rs320	T/G		intron	19829350	8
LPL	rs326	A/G	0.32	intron	19829712	8
PRKCB1	rs1015408	A/T		intron	24176913	16
SLC2A4	rs5435	C/T	0.45	syn	7387688	17
TCF2	rs2688	G/T		utr	36242481	17
TGFBR2	rs6792117	A/G		intron	30678972	3

Abbreviations are: Average Heterozygousity Value, Het; nonsynonymous amino acid substitution, nonsyn; synonymous amino acid substitution, syn; Untranslated Region, utr.

Table 5. Summary of TDT data for 23 SNPs Under Analysis.

Locus	SNP rs#	Mutation	SNP	Allele	M1	M2	Total	M1/M2	Chi-Sq	Probability
APOE	rs429358	syn	C/T	T	15	32	47	0.319	6.149	0.025
APOE	rs7412	syn	C/T	C	12	3	15	0.800	5.400	0.025
BCL2	rs1481031	intron	C/T	C	39	19	58	0.672	6.897	0.010
BCL2	rs12457700	intron	C/T	C	36	16	52	0.692	7.692	0.010
BCL2	rs2062011	intron	A/T	T	42	17	59	0.712	10.593	0.005
COL4A1	rs614282	intron	T/C	C	40	17	57	0.702	9.281	0.005
COL4A1	rs679062	intron	C/T	T	43	15	58	0.741	13.517	0.0005
GLUT4	rs5435	syn	C/T	C	39	22	61	0.639	4.738	0.05
LAMA4	rs3734287	intron	C/T	C	37	19	56	0.661	5.786	0.025
LAMC1	rs10797819	intron	G/A	A	46	28	74	0.622	4.378	0.05
LAMC1	rs4652775	intron	A/T	A	45	29	74	0.608	3.459	
LAMC1	rs2296288	syn	T/C	T	46	29	75	0.613	3.853	0.05
LAMC1	rs7556132	nonsyn	A/G	A	47	29	76	0.618	4.263	0.05
LAMC1	rs2296292	syn	A/C	A	45	28	73	0.616	3.959	0.05
LAMC1	rs20557	syn	T/C	T	46	27	73	0.630	4.945	0.05
LAMC1	rs7410919	nonsyn	T/C	T	47	29	76	0.618	4.263	0.05
LPL	rs320	intron	G/T	T	41	19	60	0.683	8.067	0.005
LPL	rs326	intron	A/G	A	41	21	62	0.661	6.452	0.025
LPL	rs13702	3'UTR	C/T	T	40	18	58	0.690	8.345	0.005
PRKCB1	rs1015408	intron	A/T	T	30	15	45	0.667	5.000	0.05
TCF2	rs2688	3'UTR	C/A	C	43	25	68	0.632	4.765	0.05
TGFBR2	rs6792117	intron	A/G	G	41	23	64	0.641	5.063	0.025
TGFBR3	hCV3130125	intron	A/C	C	42	23	65	0.646	5.554	0.025

The probability that an allelic transmission is linked to the diabetic-nephropathy phenotype is calculated using the values in columns M1, M2, and Total as described by Ewens and Spielman, 1995.

A.3 University of Pennsylvania Contribution.

The work done in Pittsburgh is duplicated at the University of Pennsylvania. This exercise will serve as a blind and efficient Q.C. of all the tests performed at both institutions.

Since the last Progress Report (10/21/03), Dr. Spielman has been extending the list of SNPs to be tested in candidate genes for DN as part of the collaboration with us. The studies carried out on family material collected at the University of Pennsylvania are hereafter reported.

To date, a total of 103 families all having at least one offspring with diabetes of long duration were studied. (Forty-six of these families are part of the HBDI collection of T1DM multiplex families). The work was previously summarized on the large collection of microsatellite markers and SNPs located in or near candidate genes, and on analysis of linkage and association using the TDT. In the past year, additional SNPs were genotyped in genes for which there was preliminary evidence for association with DN, and new candidate genes were investigated based on association and gene expression studies that appeared in the literature.

KEY RESEARCH ACCOMPLISHMENTS: A total of 103 candidate genes have now been tested for linkage disequilibrium with DN using family-based TDT analysis. The last progress report included nominally significant results found with SNPs in the AGTR1 gene region, COL4A1, LAMC1, MMP9 and TCF2. In the past year, Dr. Spielman has genotyped 202 SNPs. Some of these were additional SNPs in genes previously tested, but most were located in or near 35 new genes. The attached Table presents complete listing of the 68 genes: 35 new genes plus 33 included in the previous Progress Report for which additional SNPs have been genotyped. Among the new genes that were tested, nominally positive results were found in:

- **BCL2.** Eleven SNPs in the gene were genotyped; three SNPs located in a 24 kb region in intron 1 gave nominally significant evidence for association with DN: rs2062011 (p=0.0011), hCV1408500 (p=0.0055) and rs1481031 (p=0.0086).
- **p22phox (CYBA).** Three SNPs were genotyped in p22phox, including C242T which causes a His→Tyr change at amino acid position 72, In our collection of type 1 diabetes families, the C-allele was significantly over-transmitted (p=0.032) compared to the T-allele.
- **GLUT4.** We tested one SNP in the small gene GLUT4/SLC2A4, rs5435, Asn130Asn, a synonymous mutation in exon 4, which yielded a nominally significant result (p=0.024).
- **LAMA4.** Eight SNPs and one microsatellite were genotyped in LAMA4. One SNP, rs3734287, located in an intron, gave a nominally significant result (p=0.016).

- **LPL.** Five SNPs in LPL were tested for association with DN. Three of these SNPs located in a 5.4 kb region near the 3' end of LPL all had nominally significant TDT results: rs320 (p=0.005), rs326 (p=0.011) and rs13702 (p=0.004).
- **TGFBR.** A total of 17 SNPs were genotyped in two TGFB-receptor genes, 7 in TGFBR2 and 10 in TGFBR3. One SNP in each gene gave a nominally significant result: rs6792117 located in an intron of TGFBR2 (p=0.024) and rs12756024 located in an intron of TGFBR3 (p=0.018).
- **USF1.** Four SNPs in USF1 were genotyped. One of these, rs2516839, located in the 5'UTR gave a nominally significant result. (p=0.047).

REPORTABLE OUTCOMES:

Manuscript in preparation: Assessment of 97 Candidate Genes for Diabetic Nephropathy by TDT. K. Gogolin Ewens, R.A.V. George, L.K. Southworth, F.N. Ziyadeh, R.S. Spielman.

Abstract: Linkage and association analysis of candidate genes for diabetic nephropathy. K. Gogolin Ewens, R.A.V. George, L.K. Southworth, F.N. Ziyadeh, R.S. Spielman. Am J Hum Genet, 71 (supplement):1680, 2002.

CONCLUSIONS: Although the results described above are nominally significant, they must be considered preliminary in view of the small sample size. In order to confirm and expand these results, additional families are collected in Pittsburgh, Hawaii and at Walter Reed. In the regions of suggestive associations, Dr. Spielman will also be testing additional SNPs identified from the Celera database and public databases as well as by sequencing of interesting candidate regions.

TABLE 6. Single nucleotide polymorphisms (SNPs) in or near candidate genes for diabetic nephropathy that have been analyzed for linkage and association with the TDT since the last progress report.

DN CANDIDATE GENE	GENE SYMBOL	SNP ID	mutation		Allele	T	not T	total	%T	Chi-Sq	p
activin A receptor, type II	ACVR2	ACVR2_hCV11175435	intron	T/C	C	33	27	60	0.55	0.6	
		ACVR2_hCV7608216	intron	C/T	C	23	20	43	0.5349	0.209	
		ACVR2_hCV3144660	intron	G/T	T	31	28	59	0.525	0.153	
		ACVR2_hCV11175391	intron	G/A	A	33	27	60	0.550	0.6	
		ACVR2_hCV3144652	intron	C/G	C	30	25	55	0.545	0.455	
angiogenin, ribonuclease, RNase A family, 5	ANG	ANG_hCV2742351	intron	C/T	C	30	24	54	0.556	0.667	
B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene	BCL2	BCL2_hCV7905447	3' UTR	C/T	C	31	29	60	0.517	0.067	
		BCL2_hCV7905342	intron	T/C	T	36	30	66	0.545	0.545	
		BCL2_hCV11497042	intron	G/A	G	14	10	24	0.583	0.667	
		BCL2_hCV8685764	**intron**	**C/T**	**C**	**39**	**19**	**58**	**0.672**	**6.897**	**0.0086**
		BCL2_hCV1408500	**intron**	**C/T**	**C**	**36**	**16**	**52**	**0.692**	**7.692**	**0.0055**
		BCL2_hCV1408502	**intron**	**A/T**	**T**	**42**	**17**	**59**	**0.712**	**10.593**	**0.0011**
		BCL2_hCV1408482	intron	G/A	G	40	27	67	0.597	2.522	
		BCL2_hCV1728132	intron	G/C	G	46	31	77	0.597	2.922	
		BCL2_hCV8687299	intron	G/A	G	33	25	58	0.569	1.103	
		BCL2_hCV2855833	intron	C/T	C	32	27	59	0.542	0.424	
		BCL2_hCV2855835	4.1 kb 5'	C/A	C	38	30	68	0.559	0.941	
bone morphogenetic protein 7	BMP7	BMP7_hCV2090176	intron	A/G	A	34	28	62	0.548	0.581	
		BMP7_hCV2090207	intron	A/G	A	40	35	75	0.533	0.333	
		BMP7_hCV1364067	intron	G/A	A	25	15	40	0.625	2.5	
		BMP7_hCV1364089	intron	A/C	A	42	27	69	0.609	3.261	
bradykinin receptor B2	BDKRB2	BDKRB2_hCV11469547	intron	A/G	A	29	28	57	0.509	0.018	
		BDKRB2_hCV7565899	Cys(T)14Arg(C)	C/T	C	13	6	19	0.684	2.579	
caldesmon 1	CALD1	CALD1_hCV2808728	5' UTR	A/G	G	32	28	60	0.5333	0.267	
		CALD1_hCV348686	intron	A/C	C	33	26	59	0.5593	0.831	
		CALD1_hCV346465	intron	A/C	C	31	29	60	0.5167	0.067	
		CALD1_hCV308959	intron	C/T	T	36	28	64	0.5625	1	
chemokine (C-C motif) ligand 2	CCL2	CCL2_hCV11939405	Cys(C)35Cys(T)	C/T	T	35	28	63	0.556	0.778	
chemokine (C-C motif) receptor 5	CCR5	CCR5_rs1799987	G59029A in 5' UTR	A/T	T	33	32	65	0.508	0.015	
		CCR5_hCV2284079	3' UTR	T/G	G	31	30	61	0.508	0.016	
cysteine knot superfamily 1, BMP antagonist 1 (gremlin)	CKTSF1B1	CKTSF1B1_hCV180222	3' UTR	G/A	G	35	34	69	0.507	0.014	
Collagen 4A3	COL4A3	COL4A3_hCV408687	intron	G/T	T	39	29	68	0.5735	1.471	
		COL4A3_STRP1	intron		15	23	24	47	0.4894	0.021	
		COL4A3_hCV497868	intron	A/G	A	28	23	51	0.549	0.49	
		COL4A3_hCV381070	intron	C/T	C	35	31	66	0.5303	0.242	
		COL4A3_hCV435102	intron	A/C	A	21	20	41	0.5122	0.024	
Collagen 4A4	COL4A4	COL4A4_hCV11523963	9.3 kb 3'	A/G	G	25	22	47	0.532	0.191	
		COL4A4_hCV16171363	Val(G)1513Val(A)	C/T	C	32	28	60	0.533	0.267	
		COL4A4_hCV435282	intron	A/T	T	36	30	66	0.545	0.545	
		COL4A4_hCV388099	intron	G/A	A	32	29	61	0.525	0.148	
		COL4A4_hCV22274313	Ser(T)482Pro(G)	A/G	A	36	30	66	0.545	0.545	
		COL4A4_hCV72520	intron	T/G	G	31	27	58	0.534	0.276	
		COL4A4_hCV154148	intron	A/G	G	30	28	58	0.517	0.069	
connective tissue growth factor	CTGF	CTGF_hCV1764942	intergenic-1.5 kb 5'	G/C	G	29	26	55	0.527	0.164	
cytochrome b-245, alpha polypeptide (p22phox)	CYBA	CYBA_rs1049255	Ala(C)174Val(T)	C/T	T	33	31	64	0.5156	0.063	
		CYBA_hCV11291909	intron	C/T	C	35	34	69	0.5072	0.014	
		CYBA_hCV2038	**His(C)72Tyr(T)**	**A/G**	**G**	**32**	**17**	**49**	**0.6531**	**4.592**	**0.0321**
cathepsin D	CTSD	CTSD_hCV189639	intron	C/T	C	40	37	77	0.519	0.117	
cathepsin L	CTSL	CTSL_hCV2704846	intron	C/T	T	34	31	65	0.523	0.138	
endothelin converting enzyme 1	ECE1	ECE1_hCV2221518	intron	C/T	C	29	29	58	0.5	0	
		ECE1_D1S478	intron		14	21	21	42	0.500	0	
		ECE1_hCV2234019	0.3 kb 5'	G/T	T	30	26	56	0.5357	0.286	
		ECE1_hCV2488389	29.6 kb 5'	G/T	T	43	27	70	0.6143	3.657	
endothelin 1	EDN1	EDN1_hCV3107156	intron	A/G	G	23	20	43	0.535	0.209	
endothelin-3	EDN3	EDN3_hCV2506285	intron	C/T	C	34	31	65	0.5231	0.138	
		EDN3_hCV2506294	1.8 kb 5'	C/T	C	31	30	61	0.5082	0.016	
endothelin receptor-type A	EDNRA	EDNRA_hCV8869375	5' UTR	A/G	G	35	33	68	0.5147	0.059	
		EDNRA_STRP1	intron		9	25	28	53	0.472	0.17	
		EDNRA_hCV1736728	intron	C/T	C	29	29	58	0.5	0	
		EDNRA_hCV1736670	His(C)323His(T)	C/T	C	32	28	60	0.5333	0.267	
		EDNRA_hCV1736669	Glu(A)335Glu(G)	A/G	A	32	29	61	0.5246	0.148	
		EDNRA_hCV1736665	3' UTR	A/G	A	33	30	63	0.5238	0.143	
endothelin receptor-type B	EDNRB	EDNRB_D13S1281	6.7 kb 3'		6	32	24	56	0.571	1.143	
		EDNRB_hCV11706905	2.6 kb 3'	A/T	A	23	21	44	0.523	0.091	
		EDNRB_hCV15752350	intron	C/G	G	12	12	24	0.5	0	
		EDNRB_hCV272290	introm	C/G	C	10	8	18	0.556	0.222	
		EDNRB_hCV1923554	intron	G/A	G	33	30	63	0.5238	0.143	
epidemal growth factor	EGF	EGF_hCV8904301	intron	G/A	A	23	23	46	0.500	0	
		EGF_hCV335748	intron	A/G	A	24	23	47	0.511	0.021	
		EGF_hCV336634	intron	T/C	C	31	26	57	0.544	0.439	

DN CANDIDATE GENE	GENE SYMBOL	SNP ID	mutation		Allele	T	not T	total	%T	Chi-Sq	p
		EGF_hCV15955612	Met(G)708Ile(A)	G/A	A	31	25	56	0.554	0.643	
fibulin 1	FBLN1	FBLN1_hCV2454340	intron	G/A	A	35	31	66	0.530	0.242	
		FBLN1_hCV11879747	intron	C/G	C	41	35	76	0.5395	0.474	
		FBLN1_hCV2482867	4.4 kb 3'	A/T	T	34	32	66	0.515	0.061	
fibrillin	FBN1	FBN1_hCV2908746	3' UTR	A/G	G	27	20	47	0.5745	1.043	
		FBN1_hCV1865677	intron	A/G	G	26	19	45	0.5778	1.089	
		FBN1_hCV1865698	intron	C/T	C	31	23	54	0.5741	1.185	
		FBN1_hCV3093295	intron	C/T	T	31	26	57	0.5439	0.439	
		FBN1_hCV3224829	intron	A/G	G	27	22	49	0.551	0.51	
		FBN1_hCV25474482	intron	A/C	C	20	17	37	0.5405	0.243	
fibronectin 1	FN1	FN1_hCV71462	Tyr(T)2265Tyr(C)	A/G	G	33	26	59	0.5593	0.831	
		FN1_hCV2110676	intron	C/G	C	36	30	66	0.5455	0.545	
		FN1_hCV2110728	intron	A/G	G	37	31	68	0.5441	0.529	
v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	FOS_hCV3269911	Se(T)r84Ser(C)	C/T	C	33	29	62	0.5323	0.258	
glutamine-fructose-6-phosphate transaminase 2	GFPT2	GFPT2_hCV1391323	3' UTR	C/T	C	20	20	40	0.5	0	
		GFPT2_rs2303007	Val(G)471Ile(A)	C/T	C	20	19	39	0.5128	0.026	
growth hormone	GH1	GH1_hCV2955804	9.4 kn 5'	G/C	G	33	25	58	0.569	1.103	
glucose transporter-1, solute carrier family 2, member 1	GLUT1, SLC2A	SLC2A1_hCV16194495	Pro(C)196Pro(T)	C/T	T	28	21	49	0.5714	1	
		SLC2A1_hCV1166174	intron	A/C	A	31	22	53	0.5849	1.528	
		SLC2A1_rs841853	intron	T/G	T	26	20	46	0.565	0.783	
		SLC2A1_hCV1166185	Ala(C)15Ala(T)	G/A	A	27	21	48	0.5625	0.75	
glucose transporter-2, solute carrier family 2, member 2	GLUT2, SLC2A	SLC2A2_hCV3023463	Phe(C)479Phe(T)	A/G	A	25	24	49	0.5102	0.02	
		SLC2A2_hCV3142148	Thr(C)110Ile(T)	A/G	A	14	11	25	0.56	0.36	
		GLUT2_STRP1	intron		11	23	22	45	0.5111	0.022	
solute carrier family 2, member 4 (facilitated glucose transporter),	GLUT4,SLC2A4	**SLC2A4_hCV2552981**	**Asn(C)130Asn(T)**	**C/T**	**C**	**39**	**22**	**61**	**0.6393**	**4.738**	**0.029503**
heparan sulfate proteoglycan 1 (syndecan 2)	HSPG1, SDC2	SDC2_hCV2087148	1.2 kb 5'	C/T	T	31	22	53	0.585	1.528	
		HSPG1_D8S1018	intron		7	25	37	62	0.4032	2.323	
		SDC2_hCV2087268	intron	C/T	C	20	16	36	0.556	0.444	
		SDC2_hCV2087297	intron	A/T	T	24	22	46	0.522	0.087	
		SDC2_hCV2087211	intron	G/T	G	34	29	63	0.540	0.397	
		SDC2_hCV3223155	Tyr(T)51Tyr(C)	C/T	T	19	18	37	0.514	0.027	
		SDC2_hCV11331168	Thr(A)71Ser(T)	A/T	A	24	18	42	0.571	0.857	
heparan sulfate proteoglycan 2 (perlecan)	HSPG2	HSPG2_hCV3036142	1.1 kb 3'	A/G	A	34	21	55	0.618	3.073	
		HSPG2_hCV15966517	His(C)3211Tyr(T)	A/G	A	20	12	32	0.625	2	
		HSPG2_hCV1603698	intron	A/G	A	37	32	69	0.536	0.362	
		HSPG2_STRP1	intron		7	21	20	41	0.5122	0.024	
		HSPG2_hCV1603733	intron	C/T	T	29	28	57	0.509	0.018	
		HSPG2_hCV205528	intron	C/T	T	23	18	41	0.561	0.61	
insulin-like growth factor 1 receptor	IGF1R	IGF1R_hCV11527385	intron	C/G	C	30	27	57	0.526	0.158	
		IGF1R_hCV1599413	intron	A/G	G	28	25	53	0.528	0.17	
		IGF1R_hCV1119260	intron	C/T	C	29	22	51	0.569	0.961	
		IGF1R_hCV15910812	intron	A/G	G	31	28	59	0.525	0.153	
		IGF1R_hCV1599489	intron	C/T	C	32	24	56	0.571	1.143	
		IGF1R_hCV11713576	intron	A/G	G	26	21	47	0.553	0.532	
		IGF1R_AFMA305YE1	intron		8	28	27	55	0.509	0.018	
		IGF1R_hCV239822	intron	A/G	G	32	27	59	0.542	0.424	
		IGF1R_hCV1834836	intron	C/T	C	38	36	74	0.514	0.054	
interleukin 10	IL10	IL10_hCV8828803	intron	C/T	T	27	26	53	0.5094	0.019	
integrin, alpha 3	ITGA3	ITGA3_hCV2539685	intron	A/G	G	38	31	69	0.551	0.71	
	ITGA3	ITGA3_STRP	intron		7	23	34	57	0.404	2.123	
laminin A4	LAMA4	LAMA4_hCV2462170	Val(A)1713Val(T)	A/T	A	30	29	59	0.5085	0.017	
		LAMA4_hCV2462178	intron	C/T	T	44	32	76	0.5789	1.895	
		LAMA4_hCV2462186	**intron**	**C/T**	**C**	**37**	**19**	**56**	**0.6607**	**5.786**	**0.016154**
		LAMA4_hCV2462219	intron	A/G	G	35	31	66	0.5303	0.242	
		LAMA4_STRP1	intron		13	27	15	42	0.643	3.429	
		LAMA4_hCV2462251	His(C)491Tyr(T)	A/G	A	28	24	52	0.5385	0.308	
		LAMA4_hCV2462280	intron	A/C	A	33	30	63	0.5238	0.143	
		LAMA4_hCV2462319	intron	C/G	G	20	18	38	0.5263	0.105	
		LAMA4_hCV11903282	intron	A/G	A	33	23	56	0.5893	1.786	
lectin, galactoside-binding, soluble, 3	LGALS3	LGALS3_hCV7593627	intron	C/G	C	37	27	64	0.5781	1.563	
lipoprotein lipase	LPL	LPL_hCV9642885	intron	C/T	C	28	26	54	0.519	0.074	
		LPL_hCV1842992	Glu145Glu	G/A	A	13	11	24	0.5417	0.167	
		LPL_rs285	intron	C/T	T	35	33	68	0.5147	0.059	
		LPL_rs320	**intron**	**G/T**	**T**	**41**	**19**	**60**	**0.6833**	**8.067**	**0.005**
		LPL_hCV1843005	**intron**	**A/G**	**A**	**41**	**21**	**62**	**0.661**	**6.452**	**0.011**
		LPL_hCV901792	Ser(C)474X(G)	C/G	C	10	10	20	0.5	0	
		LPL_hCV9639448	**3' UTR**	**C/T**	**T**	**40**	**18**	**58**	**0.6897**	**8.345**	**0.004**
matrix metalloproteinase 1	MMP1	MMP1_hCV632723	3'UTR	G/A	G	40	25	65	0.6154	3.462	
		MMP1_hCV7492469	4.3 kb 5'	A/T	T	32	28	60	0.5333	0.267	
matrix metalloproteinase 2	MMP2	MMP2_hCV15872551	intron	G/C	C	32	31	63	0.508	0.016	
		MMP2_hCV3225975	intron	C/G	C	35	33	68	0.515	0.059	

DN CANDIDATE GENE	GENE SYMBOL	SNP ID	mutation		Allele	T	not T	total	%T	Chi-Sq	p
matrix metalloproteinase 3	MMP3	MMP3_hCV785964	Ala(C)362Ala(T)	C/T	C	39	34	73	0.5342	0.342	
		MMP3_hCV3047717	Glu(G)45Lys(A)	G/A	G	39	33	72	0.5417	0.5	
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	NFKB1_hCV3066487	intron	A/G	A	33	26	59	0.5593	0.831	
		NFKB1_hCV804243	intron	A/G	G	29	21	50	0.58	1.28	
		NFKB1_hCV804225	intron	A/G	G	30	19	49	0.6122	2.469	
		NFKB1_hCV3066444	intron	C/G	C	32	24	56	0.5714	1.143	
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	NFKB2	NFKB2_hCV1777528		A/G	G	17	15	32	0.5313	0.125	
		NID_STRP1	intron								
nidogen (enactin)	NID	NID_hCV16179265	intron	C/G	G	32	25	57	0.5614	0.86	
		NID_hCV8860646	intron	C/T	T	19	16	35	0.5429	0.257	
platelet-derived growth factor-β	PDGFB	PDGFB_hCV15962706	intron	G/T	G	35	32	67	0.522	0.134	
		PDGFB_hCV15872223	intron	C/T	T	27	22	49	0.551	0.51	
		PDGFB_STRP1	5' UTR		5	18	26	44	0.409	1.455	
platelet-derived growth factor receptor, β	PDGFRB	PDGFRB_hCV11263234	7.6 kb 3'	A/G	G	35	34	69	0.5072	0.014	
		PDGFRB_hCV3220603	intron	A/G	G	41	33	74	0.5541	0.865	
		PDGFRB_hCV344379	1.9 kb 5'	A/G	A	40	27	67	0.597	2.522	
peroxisome proliferative activated receptor, gamma	PPARG	PPARG_hCV9384508	intron	T/C	C	36	33	69	0.5217	0.13	
		PPARG_hCV9384377	intron	G/T	T	31	23	54	0.5741	1.185	
		PPARG_hCV1129864	Ala(G)12Pro(C)	G/C	C	22	18	40	0.55	0.4	
		PPARG_hCV1129842	intron	C/T	C	40	34	74	0.5405	0.486	
		PPARG_hCV1129824	intron	A/G	G	34	28	62	0.5484	0.581	
		PPARG_hCV3158666	intron	G/A	A	39	34	73	0.5342	0.342	
renin	REN	REN_hCV8687919	intron	G/T	T	29	26	55	0.527	0.164	
SA hypertension-associated homolog (rat)	SAH	SAH_hCV2192241	intron	G/T	G	23	21	44	0.523	0.091	
selectin E	SELE	SELE_hCV2459451	intron	C/T	T	31	29	60	0.5167	0.067	
		SELE_hCV8919523	Tyr(T)468His(C)	C/T	A	12	11	23	0.5217	0.043	
selectin L	SELL	SELL_hCV11975318	intron	G/A	A	27	26	53	0.5094	0.019	
serum/glucocorticoid regulated kinase	SGK	SGK_hCV1347310	8.2 kb 5'	C/T	C	36	29	65	0.554	0.754	
solute carrier family 9 (Na+/H+ antiporter)	SLC9A1	SLC9A1_hCV12009155	3' UTR	A/G	G	34	32	66	0.5152	0.061	
		SLC9A1_STRP1	intron		8	25	35	60	0.417	1.667	
		SLC9A1_hCV1900526	intron	C/T	C	35	31	66	0.5303	0.242	
solute carrier family 12 (sodium/chloride transporters), member 3	SLC12A3	SLC12A3_hCV7729434	intron	C/T	C	27	26	53	0.5094	0.019	
		SLC12A3_rs11643718	Gln(A)913Arg(G)	A/G	G	11	8	19	0.5789	0.474	
		SLC12A3_hCV11196576	2.9 kb 3'	A/G	A	39	29	68	0.5735	1.471	
transforming growth factor B1	TGFB1	TGFB1_hCV7818385	5.6 kb 3'	T/C	T	16	14	30	0.533	0.133	
		TGFB1_3239 (pyro)	Ile263Thr	G/A	G	6	4	10	0.600	0.4	
		TGFB1_hCV22272997	Pro(C)10Leu(T)	A/G	A	23	21	44	0.523	0.091	
		TGFB1_3236ps	C-509T in 5' UTR	A/G	G	24	24	48	0.500	0	
transforming growth factor B2	TGFB2	TGFB2_hCV12081791	intron	C/T	C	34	30	64	0.5313	0.25	
		TGFB2_STRP1	intron	no allele >40 transmissions							
		TGFB2_hCV12081803	intron	A/G	G	22	21	43	0.5116	0.023	
transforming growth factor-B receptor2	TGFBR2	TGFBR2_hCV3158972	intron	A/C	C	41	31	72	0.5694	1.389	
		TGFBR2_hCV11565979	intron	A/T	T	34	30	64	0.5313	0.25	
		TGFBR2_hCV1612549	intron	A/G	G	34	32	66	0.5152	0.061	
		TGFBR2_hCV972343	intron	A/G	G	24	22	46	0.5217	0.087	
		TGFBR2_hCV8778179	intron	A/G	G	27	21	48	0.5625	0.75	
		TGFBR2_hCV1612506	**Intron**	**A/G**	**G**	**41**	**23**	**64**	**0.6406**	**5.063**	**0.024442**
		TGFBR2_hCV1612485	intron	A/T	A	19	16	35	0.5429	0.257	
		TGFBR2_hCV1612480	2.8 kb 3'	A/G	A	29	25	54	0.537	0.296	
		TGFBR3_hCV8368244	3' UTR	C/T	T	19	15	34	0.5588	0.471	
transforming growth factor-B receptor3	TGFBR3	TGFBR3_hCV945103	silent mutation no deta	A/G	A	10	5	15	0.6667	1.667	
		TGFBR3_hCV1931721	Phe(C)673Phe(T)	A/G	G	38	30	68	0.5588	0.941	
		TGFBR3_hCV16195006	Ser(A)171Ser(G)								
		TGFBR3_hCV3130156	intron	A/C	A	38	32	70	0.5429	0.514	
		TGFBR3_hCV3130147	intron	A/T	T	37	29	66	0.5606	0.97	
		TGFBR3_hCV3130125	**Intron**	**A/C**	**C**	**42**	**23**	**65**	**0.6462**	**5.554**	**0.0184**
		TGFBR3_hCV11643684	intron	A/C	A	38	34	72	0.5278	0.222	
		TGFBR3_hCV11643667	intron	A/G	G	38	28	66	0.5758	1.515	
		TGFBR3_hCV1931638	intron	A/G	A	34	30	64	0.5313	0.25	
		TGFBR3_hCV11371756	5' UTR (or exon 3)	C/T	T	11	8	19	0.5789	0.474	
		TGFBR3_hCV3130092	intron	A/G	A	32	32	64	0.5	0	
		TGFBR3_hCV3181378	intron	A/G	A	37	35	72	0.5139	0.056	
		TGFBR3_D1S1588	intron		10	17	28	45	0.378	2.689	
thrombospondin	THBS1	THBS1_hCV3100556	missense mutation no	C/T	C	17	6	23	0.739	5.261	
		THBS1_rs2292305	Ala(G)523Thr(A)	A/G	A	16	6	22	0.7273	4.545	
		THBS1_rs3743125	3'UTR	A/G	G	17	6	23	0.7391	5.261	

DN CANDIDATE GENE	GENE SYMBOL	SNP ID	mutation		Allele	T	not T	total	%T	Chi-Sq	p
tumor necrosis factor (ligand) superfamily, member 6	TNFSF6, FasL	TNFSF6_hCV3175435	intron	G/A	G	39	35	74	0.527	0.216	
tumor necrosis factor receptor 1 precursor	TNFRSF1A	TNFRSF1A_hCV2645708	intron	A/G	G	37	29	66	0.5606	0.97	
transforming growth factor beta-stimulated protein TSC-22	TSC22, TGFB1	TSC22_hCV1925760	3.2 kb 3'	C/T	C	33	33	66	0.500	0	
		TSC22_hCV25755777	intron	C/T	T	8	6	14	0.571	0.286	
ubiquitin A-52 residue ribosomal protein fusion product 1.	UBA52	UBA52_hCV2580747	intron	C/T	T	36	35	71	0.507	0.014	
unc-13 homolog B (C. elegans)	UNC13B	UNC13B_hCV2754761	intron	C/T	C	29	28	57	0.5088	0.018	
		UNC13B_hCV1755997	intron	A/C	A	28	26	54	0.5185	0.074	
		UNC13B_hCV1756005	intron	A/T	A	33	27	60	0.55	0.6	
		UNC13B_hCV2704288	intron	C/T	C	25	23	48	0.5208	0.083	
		UNC13B_hCV2704302	intron	A/G	A	21	19	40	0.525	0.1	
		UNC13B_hCV15877163	intron	C/T	C	26	23	49	0.5306	0.184	
upstream transcription factor 1	USF1	USF1_hCV1459759	3' UTR	A/G	G	25	24	49	0.510	0.02	
		USF1_rs2073658	intron	C/T	C	22	19	41	0.537	0.22	
		USF1_hCV15949520	intron	C/G	G	22	21	43	0.512	0.023	
		USF1_hCV1839183	**5'UTR**	**C/T**	**T**	**45**	**28**	**73**	**0.616**	**3.959**	**0.046621**
upstream transcription factor 2	USF2	USF2_hCV2604928	intron	A/G	A	34	33	67	0.507	0.015	
		USF2_hCV2604934	3'UTR	C/T	C	20	16	36	0.556	0.444	
vascular endothelial growth factor	VEGF	VEGF_STRP1	3.8 kb 5'		6	23	22	45	0.511	0.022	
		VEGF_hCV1647373	intron	T/C	T	38	32	70	0.543	0.514	
kinase insert domain receptor (a type III receptor tyrosine kinase)	VEGFR2, KDR	KDR_hCV1673874	intron	C/T	C	29	21	50	0.580	1.28	
		KDR_hCV3065122	intron	C/T	C	31	23	54	0.574	1.185	
		KDR_hCV1673851	intron	A/G	G	46	37	83	0.554	0.976	

TABLE 7. Summary of TDT analysis of 202 SNPs in 68 candidate genes genotyped since the last Progress Report										
DN CANDIDATE GENE	GENES ID	# SNPs genotyped	SNPs with evidence for LD[a]	polymorphism	TDT results for SNPs showing evidence of LD[a] allele	T[b]	nT[c]	% T[b]	χ^2	p
activin A receptor, type II	ACVR2	5								
angiogenin, ribonuclease, RNase A family, 5	ANG	1								
B-cell leukemia/lymphoma 2 (bcl-2) proto-oncogene	BCL2	11	BCL2_hCV8685764	intron	C	39	19	0.6724	6.8970	0.0086
			BCL2_hCV1408500	intron	C	36	16	0.6923	7.6920	0.0055
			BCL2_hCV1408502	intron	T	42	17	0.7119	10.5930	0.0011
bone morphogenetic protein 7	BMP7	4								
bradykinin receptor B2	BDKRB2	2								
caldesmon 1	CALD1	4								
chemokine (C-C motif) ligand 2	CCL2	1								
chemokine (C-C motif) receptor 5	CCR5	2								
cysteine knot superfamily 1, BMP antagonist 1 (gremlin)	CKTSF1B1	1								
Collagen 4A3	COL4A3	4								
Collagen 4A4	COL4A4	7								
connective tissue growth factor	CTGF	1								
cytochrome b-245, alpha polypeptide (p22phox)	CYBA	3	CYBA_hCV2038	His(C)72Tyr(T)	G	32	17	0.6531	4.5920	0.0321
cathepsin D	CTSD	1								
cathepsin L	CTSL	1								
endothelin converting enzyme 1	ECE1	3								
endothelin 1	EDN1	1								
endothelin-3	EDN3	2								
endothelin receptor-type A	EDNRA	5								
endothelin receptor-type B	EDNRB	4								
epidemal growth factor	EGF	4								
fibulin 1	FBLN1	3								
fibrillin	FBN1	6								
fibronectin 1	FN1	3								
v-fos FBJ murine osteosarcoma viral oncogene homolog	FOS	1								
glutamine-fructose-6-phosphate transaminase 2	GFPT2	2								
growth hormone	GH1	1								
glucose transporter-1, solute carrier family 2, member 1	GLUT1, SLC2A1	4								
glucose transporter-2, solute carrier family 2, member 2	GLUT2, SLC2A2	3								
solute carrier family 2, member 4 (facilitated glucose transporter)	GLUT4,SLC2A4	1	SLC2A4_hCV2552981	Asn(C)130Asn(T)	C	39	22	0.6393	4.7380	0.0295
heparan sulfate proteoglycan 1 (syndecan 2)	HSPG1, SDC2	6								
heparan sulfate proteoglycan 2 (perlecan)	HSPG2	5								
insulin-like growth factor 1 receptor	IGF1R	9								
interleukin 10	IL10	1								
integrin, alpha 3	ITGA3	1								
laminin A4	LAMA4	8	LAMA4_hCV2462186	intron	C	37	19	0.6607	5.7860	0.0162
lectin, galactoside-binding, soluble, 3	LGALS3	1								
lipoprotein lipase	LPL	7	LPL_rs320	intron	T	41	19	0.6833	8.0670	0.0045
			LPL_hCV1843005	intron	A	41	21	0.6613	6.4520	0.0111
			LPL_hCV9639448	3' UTR	T	40	18	0.6897	8.3450	0.0039
matrix metalloproteinase 1	MMP1	2								
matrix metalloproteinase 2	MMP2	2								

Appendix: Table of TDT results for 202 SNPs genotyped in 68 candidate genes for diabteic nephroapthy.

DN CANDIDATE GENE	GENES ID	# SNPs genotyped	SNPs with evidence for LD[a]	polymorphism	TDT results for SNPs showing evidence of LD[a]					
					allele	T[b]	nT[c]	% T[b]	χ^2	p
matrix metalloproteinase 3	MMP3	2								
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	NFKB1	4								
nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	NFKB2	1								
nidogen (enactin)	NID	2								
platelet-derived growth factor-β	PDGFB	2								
platelet-derived growth factor receptor-β	PDGFRB	3								
peroxisome proliferative activated receptor, gamma	PPARG	6								
renin	REN	1								
SA hypertension-associated homolog (rat)	SAH	1								
selectin E	SELE	2								
selectin L	SELL	1								
serum/glucocorticoid regulated kinase	SGK	1								
solute carrier family 9 (Na+/H+ antiporter)	SLC9A1	2								
solute carrier family 12 (sodium/chloride transporters), member 3	SLC12A3	3								
transforming growth factor B1	TGFB1	4								
transforming growth factor B2	TGFB2	2								
transforming growth factor-B receptor2	TGFBR2	8	TGFBR2_hCV1612506	intron	G	41	23	0.6406	5.0630	0.0244
transforming growth factor-B receptor3	TGFBR3		TGFBR3_hCV3130125	intron	C	42	23	0.6462	5.5540	0.0184
thrombospondin	THBS1	3								
tumor necrosis factor (ligand) superfamily, member 6	TNFSF6, FasL	1								
tumor necrosis factor receptor 1 precursor	TNFRSF1A	1								
transforming growth factor beta-stimulated protein TSC-22	TSC22, TGFB1I4	2								
ubiquitin A-52 residue ribosomal protein fusion product 1.	UBA52	1								
unc-13 homolog B (C. elegans)	UNC13B	6								
upstream transcription factor 1	USF1	4	USF1_hCV1839183	5'UTR	T	45	28	0.6164	3.9590	0.0466
upstream transcription factor 2	USF2	2								
vascular endothelial growth factor	VEGF	1								
kinase insert domain receptor (a type III receptor tyrosine kinase)	VEGFR2, KDR	3								

[a] LD linkage disequilibrium

[b] T number of transmissions of the designated allele from heterozygous parents to affected offspring

[c] nT number of transmissions of the alternate allele from heterozygous parents to affected offspring

B. GENETIC INFORMATION FOR TESTING –DIABETES (GIFT-D).

GIFT-D is the educational component of our program. This current feasibility study is conducted utilizing siblings of children with diabetes who are seen at the Children's Hospital of Pittsburgh (CHP) Diabetes Center, and if effective, could be applicable to the general population. *(This feasibility study will be referred to as Phase 1.)*

The major goals of this study are to:

1) improve our ability to characterize the risk of developing type 1 diabetes (T1D) (using a risk algorithm that has been validated);
2) develop, implement, and evaluate the process and outcome of an interactive internet-based educational/counseling program (for risk notification and genetic counseling) that will communicate information about genetics and T1D risk status to health care professionals (HCP), parents, and children. This program should enhance the understanding of genetic testing for T1D in children, thus enabling the families to make a more informed decision regarding whether or not to receive the test. Three separate programs (HCP, parent, and child) are being specifically developed for the Internet;
3) evaluate the psychosocial and behavioral impact (outcomes) of receiving T1D risk information and notification of T1D risk status;
4) serve as a feasibility study for Phase 2 (general population clinical trial), which will be conducted on military bases on military dependants and their parents without T1D.

The following will detail our progress and accomplishments in achieving the above goals.

Obtaining IRB approval has been a time consuming process that has greatly impeded the progress of our work. Despite these barriers, we have forged ahead in completing the proposed programs.

1) **Improve our ability to characterize the risk of developing type 1 diabetes (using a risk algorithm that has been validated).**

   The risk algorithm for both the pilot and Phase 1 is ready to go.

2) **Develop, implement, and evaluate the process and outcome of an interactive internet-based educational/counseling program (for risk notification and genetic counseling) that will communicate information about genetics and T1D risk status to health care professionals (HCP), parents, and children. This program should enhance the understanding of genetic testing for T1D in children, thus enabling the families to make a**

more informed decision regarding whether or not to receive the test. Three separate programs (HCP, parent, and child) will be specifically developed for the Internet.

Diversified Services, Inc. (DSI), a Division of the University of Pittsburgh Medical Center, has completed the internet-based infrastructure that supports our educational/ counseling programs, evaluational measures, and data-tracking/management/storage systems. DSI is ready to open and support this website as soon as we are ready to see subjects.

Our team, along with the University Of Pittsburgh Center for Instructional Development and Distance Education (CIDDE) completed the development of the child and parent programs. A complete voice over by Nichole Johnston Baker, Miss America 1999, was added to the child's program.

Two of the three modules for the health care professionals (HCP) have been completed and the third is currently being designed with the assistance of Yvette Conley, PhD and is expected to be totally completed by January 2005. (See Appendix 2 for the transcripts) A "Collection Copyright" (through the Library of Congress) will be sought upon completion for the entire internet-based system, including all programs and both the new and modified measures.

To develop the content for the genetic education modules for families with type 1 diabetes and health professionals, we began with an extensive search of the Internet and the literature. The idea was not to 're-invent the wheel' as we developed our programs, but to glean from the observable strengths and avoid the weaknesses in existing genetic education materials. As a result, using the Miteretek Systems, Health Information Technology Institute's (HITI) criteria of accurate, relevant, and up-to-date information, we identified a number of web sites and journal articles that will serve as excellent Internet links for our genetic education programs. These listings themselves are a valuable resources for health professionals and the lay public who wish to learn more about genetics and type 1 diabetes. Thus, to share these materials with a broader audience, we submitted two manuscripts for publication that are currently In Press in *Diabetes Educator.* These are entitled "Genetics and Type 1 Diabetes: Online Resources for Diabetes Educators" by Eric R. Manthei, B.S., et al and " A Review of Education; Genetics Resources for Type 1 Diabetes on the Internet: Where Can Diabetes Educators and the General Public Access Genetic Information Online?" by Angela Feathers, B.S. et al. Copies of both manuscripts are included in Appendix 2.

3) Evaluate the psychosocial, and behavioral impact (outcomes) of receiving T1D risk information and notification of T1D risk status.

The *major outcomes* of interest are: deciding to receive genetic testing, (behavior), changes in the level of anxiety and/or depression. Parent and child versions of all the measures have been selected, created or modified, as necessary. All the measures have been loaded onto the website and permission has been obtained from the originators of the measures.

4) **Serve as a feasibility study for Phase 2 (general population clinical trial) which will be conducted on military bases on military dependants and their parents without T1D.**

In our original proposal, the genetic education/counseling program was to be implemented and evaluated in military populations on bases. As a result of the Iraq conflict, military personal were deployed and it was necessary to change the target population of the feasibility study. Rather than focusing on the "outcomes" of our program in a general military population, and given our shortened timeframe, we concentrated our efforts on the development of the internet-based programs and methods of evaluation. In addition, to expedite the preliminary trial phase, we selected a convenience sample of children and families who have a pre-existing understanding of type 1 diabetes. The Children's Hospital of Pittsburgh Diabetes Center supports an active clinic in the Johnstown community approximately 100 miles outside of Pittsburgh. Because of the distance, it offered the opportunities to provide a remote location to test telemedicine techniques, a study population with limited exposure to study recruitment and a staff ready to accommodate a research project. Families with children already diagnosed with diabetes also afforded the opportunity for quick access to facilitate the study and had the basic information on diabetes care so that the project content could be targeted specifically to genetics.

Although Phase 2 is beyond the scope of our current project, we are developing a 'product' for families that will be available to 'pilot' in the general population, such as in the military. Even though we will need to modify the education modules, the same measures and procedure methods used in out current study will be applied to evaluate the psychosocial and behavioral impact of receiving T1D risk information in a general military population. Therefore, the products that have already been developed can be modified for whatever target population necessary.

Challenges/Solutions:

1. Institutional Review Process

We have faced numerous challenges in obtaining final approval to proceed with this project. Initially, we submitted our protocol The Children's Hospital of Pittsburgh Human Rights Committee since our study is funded through and conducted in a Children's Hospital facility. This review resulted in the addition of the Pilot Study as discussed below in # 2. After this approval was granted, we were informed that we did need to submit to the DoD IRB as well. This was submitted in February 04 and returned to us in August 04, with changes. Final DoD approval is currently pending until we received our local IRB's approval. (During the time we were being reviewed at the DoD, the Children's Hospital of Pittsburgh's HRC merged into the University of Pittsburgh's IRB which now became our IRB of record). Having made the changes requested by the DoD, we submitted to the University of Pittsburgh's IRB for modifications (and a

renewal which was due 9/30/2004). As soon as we receive the University of Pittsburgh IRB approval, we will forward it to the DoD so final approval can be granted.

2. Pilot

The Belmont Report states that a new intervention must be pilot tested in an adult population prior to conducting the study on children where the psychological risks of an intervention are unknown.

As we reported last year in our Annual Report, during our initial IRB review, the Children's Hospital of Pittsburgh Human Rights Committee (HRC) cited the Belmont Report and stated that before our study of children could be approved, it would be necessary to conduct a pilot study with young adults.

The Young Adult Pilot Study include 20 young adult males and females, between the ages of 18-25 yrs old, who have a sibling with T1D. The major purpose of this pilot study is to identify the potential for adverse psychological reactions (i.e., significant clinical scores on the depression and/or anxiety scales) in an adult population receiving information regarding one's own risk of DM. Predicated on both the successful completion and results of this Young Adult Pilot Study, we will proceed with conducting the Children's Study at CHP.

Key Research Accomplishments

- Abstracts:

  Genetic Education Programs for Health Professionals: Opportunities Using Internet Technology. Submitted to the American Telemedicine Association

  National Institute of Nursing Research; Linking the Double Helix with Health: Genetics in Nursing Research Poster session: Designing an Interactive Website for Nurses to Meet the Genetic Education Needs of Patients and Their Families; Angela Feathers, Presenter

  National Institute of Nursing Research; Linking the Double Helix with Health: Genetics in Nursing Research Poster Session: Internet Based Education for Nurses; Eric Manthei, Presenter

- Manuscripts

  Dorman JS. Genetics and Diabetes. Invited Manuscript for the World Health Organization (WHO) Genomic Resource Centre, Geneva Switzerland. Submitted October 2004.

  Manthei, E.R., B.S., Siminerio, L., R.N., PhD., Conley, Y., M.S., PhD.,

Charron-Prochownik, D., R.N., PhD., Feathers, A., B.S., Dorman, J., PhD. Genetics and Type 1 Diabetes: Online Resources for Diabetes Educators. In Press: *Diabetes Educator*

Feathers, A., B.S., Manthei, E.R.B.S., Charron-Prochownik, D., R.N., PhD., Siminerio, L., R.N., PhD., Dorman, J., PhD. A Review of Educational Genetic Resources for Type 1 Diabetes on the Internet: Where Can Diabetes Educators and the General Public Access Genetic Information Online? In Press: *Diabetes Educator*

- Thesis for Masters of Science Degree in Genetic Counseling

  An Interactive Web-based Program to Educate Patients, Their Families, and the Public About the Genetics of Type 1 Diabetes, Angela Feathers, April 2004

  An Interactive Web-based Program to Educate Health Professionals About the Genetics of Type 1 Diabetes. Eric Manthei, August 2004

- Invited Presentations:

  The 2nd. Croatian Congress on Telemedicine in May, 2004.Genetic Testing For Type 1 Diabetes: Translating Results to the Community Via the Web. Presenter: Siminerio

  Presentation to the University of Hawaii and the U.S. Army, 2/10/04 Presenters: Charron-Prochownik, Dorman, Poole, and Ryan. and Siminerio Genetic Testing For Type 1 Diabetes: Translating Results to the Community Via the Web.

  Presentation to the University of Pittsburgh Endocrine Division "University-Wide Endocrine Conference" Presenters: Charron-Prochownik, Conley, Dorman, Ryan, and Siminerio.

  Children's Hospital of Pittsburgh Endocrine Conference. Presenters: Charron-Prochownik, Dorman, and Siminerio

  University of Michigan, Graduate School of Public Health Conference entitled "The Challenge Ahead: Implications of Genomics for Health Behavior and Health Education". Presentation titled, *Genetic Information for Testing Diabetes (GIFT-D) Developing a Theory-based Web Education and Counseling Program.* October 14, 2004. Presenters: Charron-Prochownik and Dorman. A manuscript is in preparation at the request of the organizers. (see request letter in Appendix 2)

- Poster Presentation – See abstracts above of Angela Feathers and Eric Manthei
- Development of two modules of the Education Program for Health Care Professional. Transcripts in Appendix 2.
- Completion of the Web Site (www.gift-d.org)
- Adding Voiceover to the Children's educational and counseling programs
- Creation of 6 age specific Assent forms

Reportable outcomes:

Our statistician, (Susan Sereika, PhD) has developed a plan to manage the data, during our preparation for data collection. She has met with DSI regarding the structuring of the data, and has met with the team regarding the redesign (Pilot) of the study. She has also rewritten the analysis and sample size sections of the study protocol. (See "Protocol" in Appendix 2)

In conclusion, although much of this year has been spent submitting and waiting for IRB approval, we were able to complete and enhance our programs and website. We are ready to conduct the pilot study, and baring any adverse psychological reactions, we will begin Phase One at the CHP Diabetes clinic early in 2005.

DAMD17-01-1-0009

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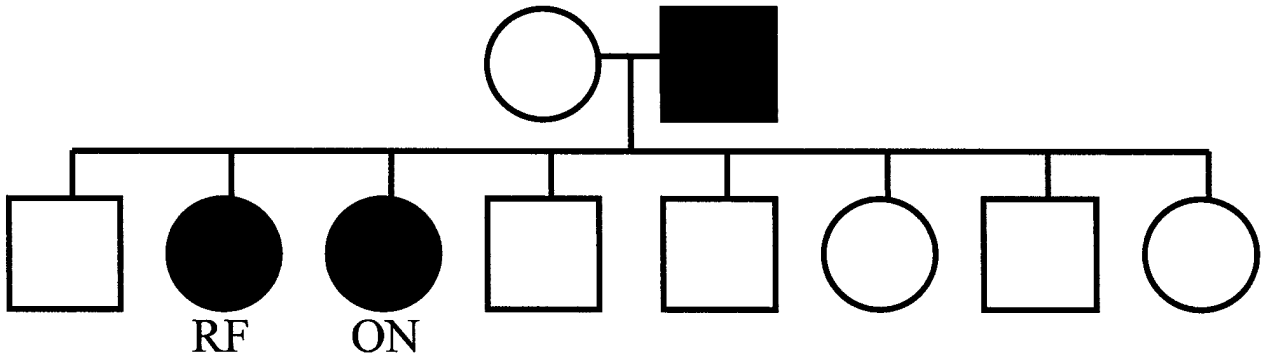
APPENDIX 1

FIGURES (AND FOOTNOTE OF EACH FIGURE)

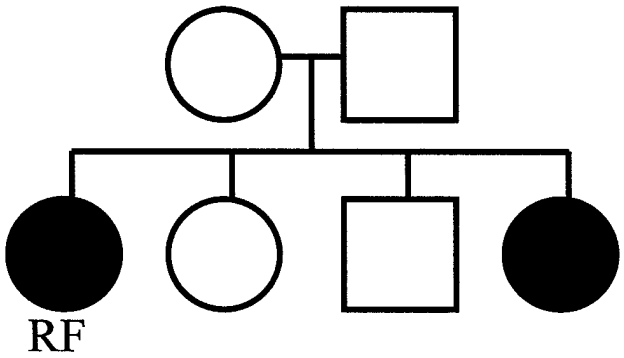
Figure 1. An example of 2 of the diabetic-nephropathy family pedigrees available as lymphoblastoid cell lines from the Children's Hospital of Pittsburgh collection of diabetic families.

Children's Hospital of Pittsburgh Collection
Diabetic-Nephropathy Pedigrees

Pedigree 0533



Pedigree 5507



Symbol	Description
Open	Unaffected
Black	Diabetic
RF	Renal Failure
ON	Overt Nephropathy

Figure 2. Illustrated description of the computer server hosting the SOP^3 database and software application consisting of human genome and SNP databases along with graphical user interface.

SOP³ Software Application Flowchart

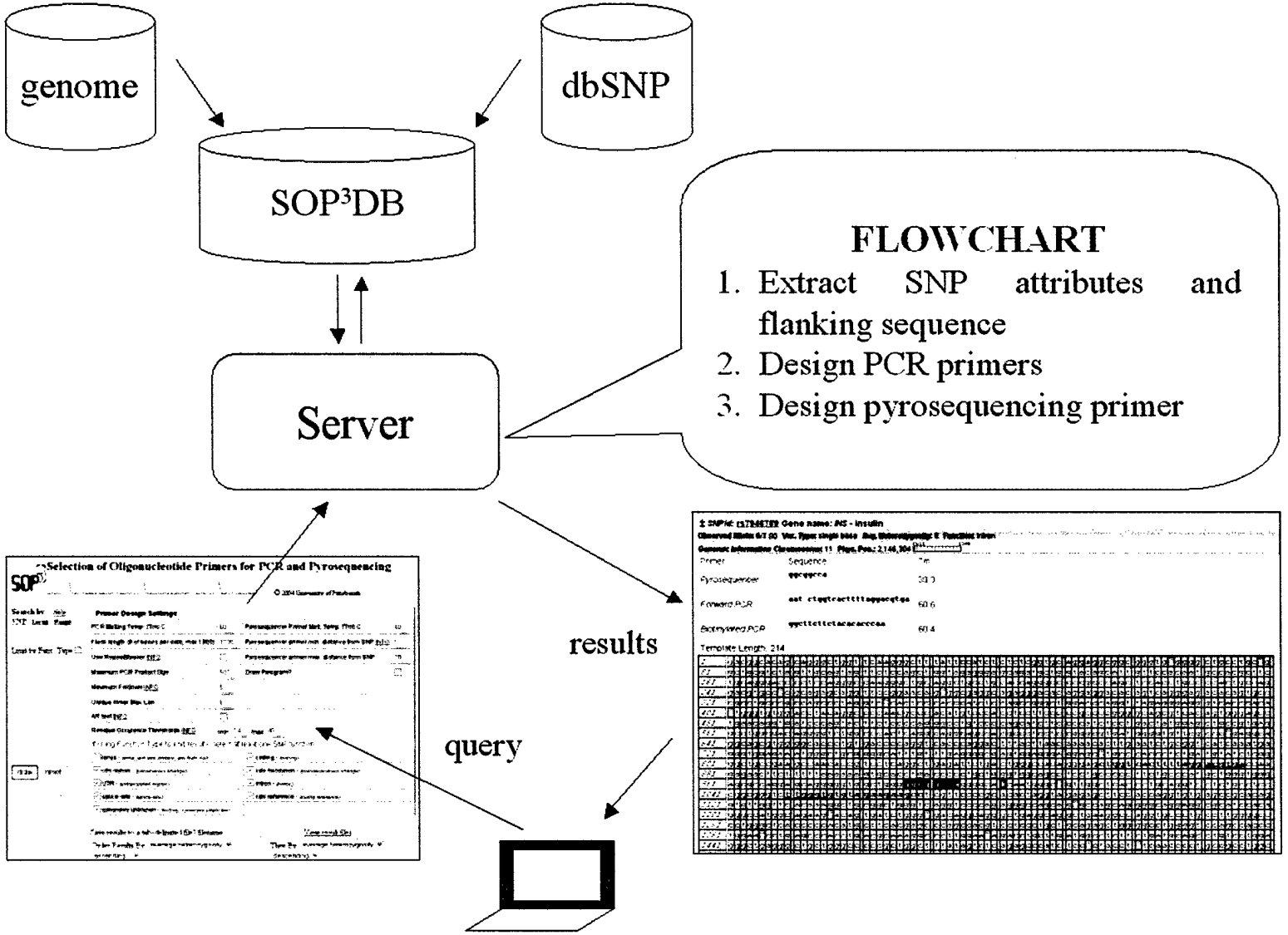


Figure 3. Output of the SOP3 application for SNP rs7946759 contained within the insulin locus. The lower part of the figure shows the chromosomal location of the SNP along with suggested oligonucleotide primers for PCR and pyrosequencing.

Output of the SOP^3 Software Application

± *SNPid:* rs7946759 **Gene name:** *INS* - insulin

Observed Allele: G/T (K) **Var. Type:** single base **Avg. Heterozygosity:** 0 **Function:** intron

Genomic Information **Chromosome:** 11 **Phys. Pos.:** 2,146,304

Primer	Sequence	Tm
Pyrosequencer	ggcggcca	38.3
Forward PCR	aat ctggtcacttttaggacgtga	60.6
Biotinylated PCR	cgaggcttcttctacacac	60.2

Template Length: 217

```
1    gacggcacag cagggctggt tcaagggctt tattccatct ctctcggtgc aggaggcggc gggtg[t]gggg ctgcctgc[t]g
81   gctgcgtcta gttgcagtag ttctccagct ggtagaggga gcagatgctg gtacagcatt gttccacaat gccacgcttc
161  tgcagggacc cctccagggc caagggctgc aggctgcctg caccagggcc cccgcccagc tccacctgcc ccactgccag
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1841 cacacccctg t[c]cccacacc cct[t]tcccca [a]acccctgtc cccaggaccc ctgtccccac acccctgtcc ccagacccct
1921 gtccccacac ccctgtcccc acacccctgt ccccacaccc ctgtcccccg gacccctgtc cccagacccc tgtccccaca
2001 c
```

Figure 4. Results of PCR amplification of genomic DNA for various SNPs performed along a gradient of PCR annealing conditions, from 53C to 64C.

PCR Temperature Gradient dU-Containing Product

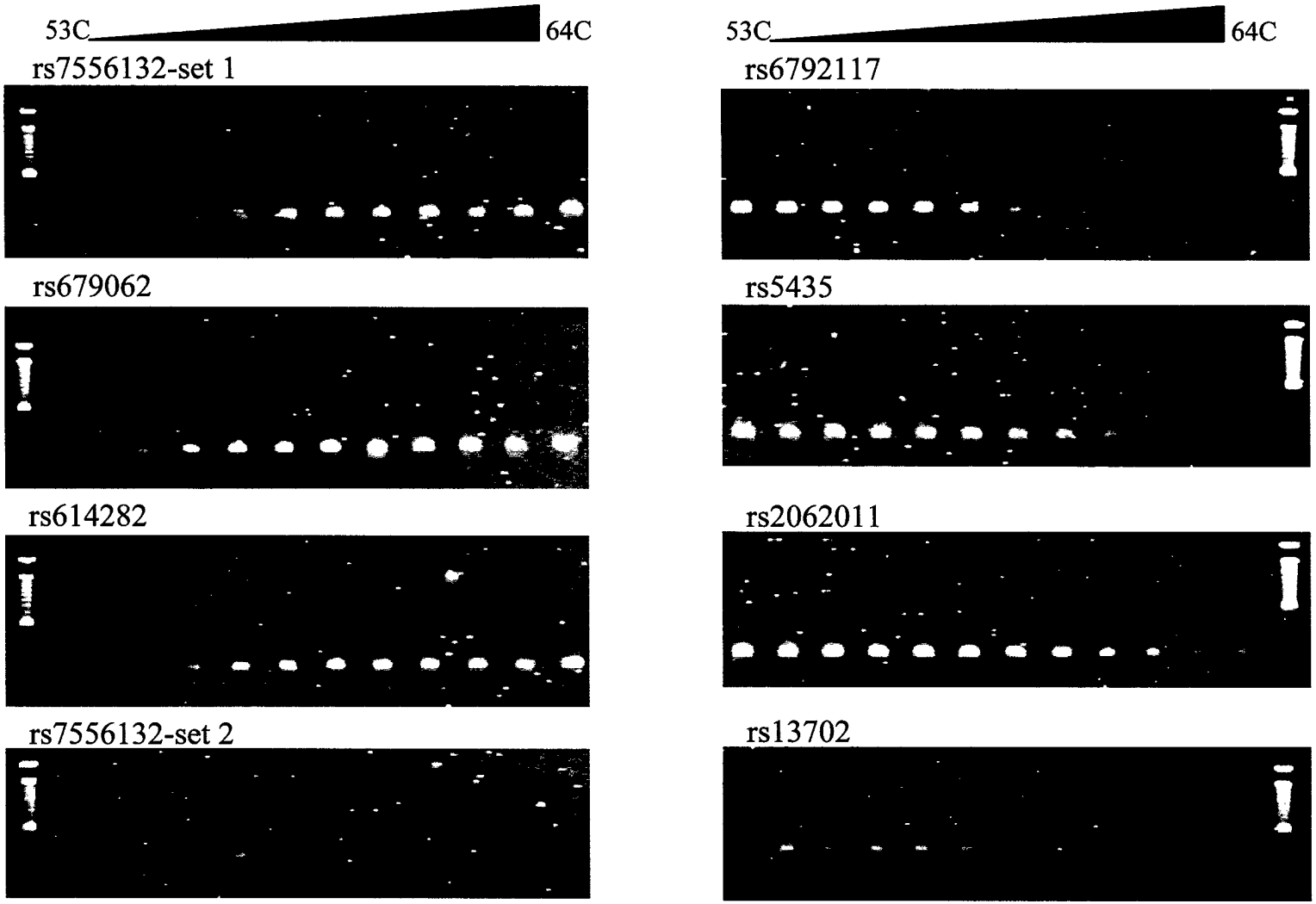


Figure 5. Description of dU-PCR protocol for minimizing cross-contamination of amplified genomic DNA samples.

Cleavage of dUMP-Containing PCR Products

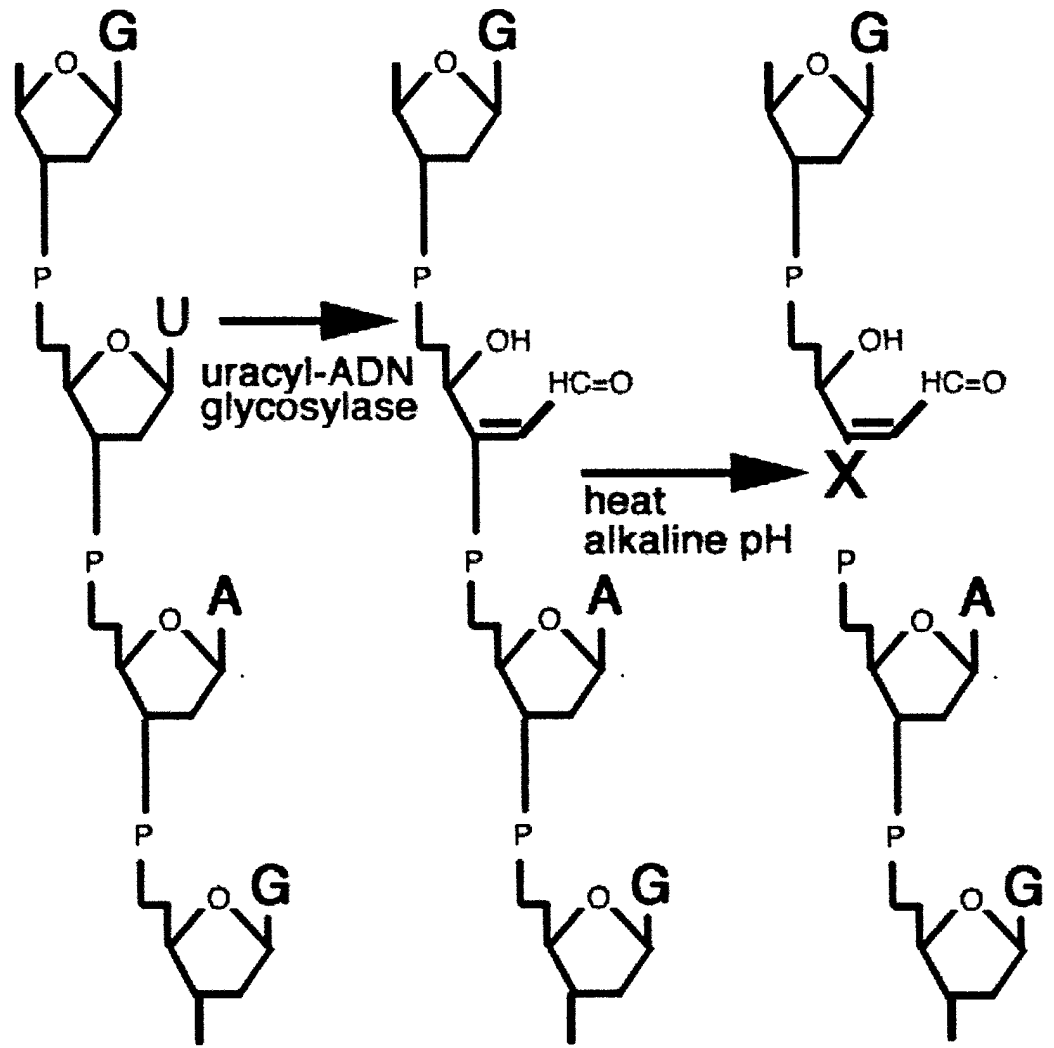
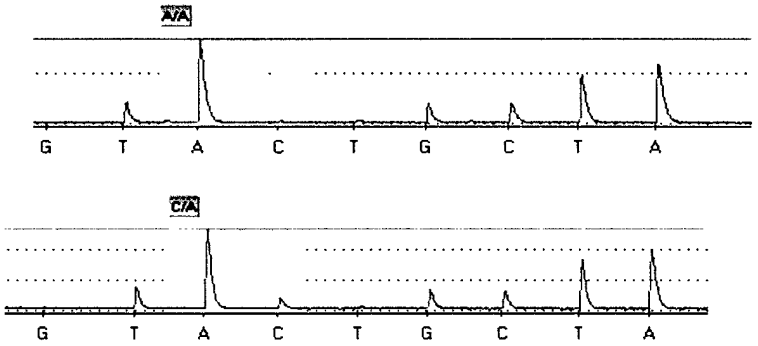


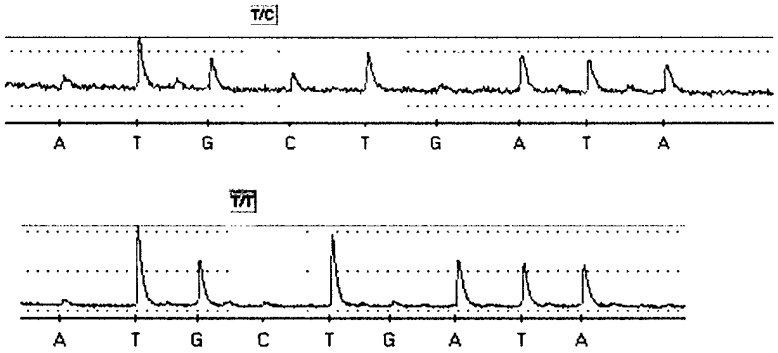
Figure 6. Pyrosequence-Based Typing results for 3 candidate SNPs associated with diabetic-nephropathy. The genotypes are indicated in the box above each line of sequencing data.

Genotyping by Pyrosequence-Based Typing

LPL (rs320)



LPL (rs13702)



PRKCB1 (rs1015408)

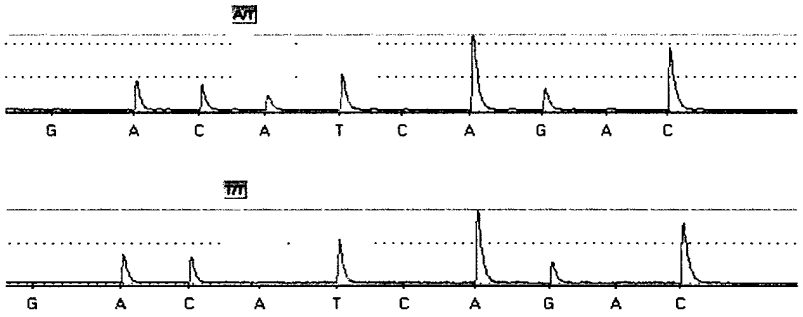
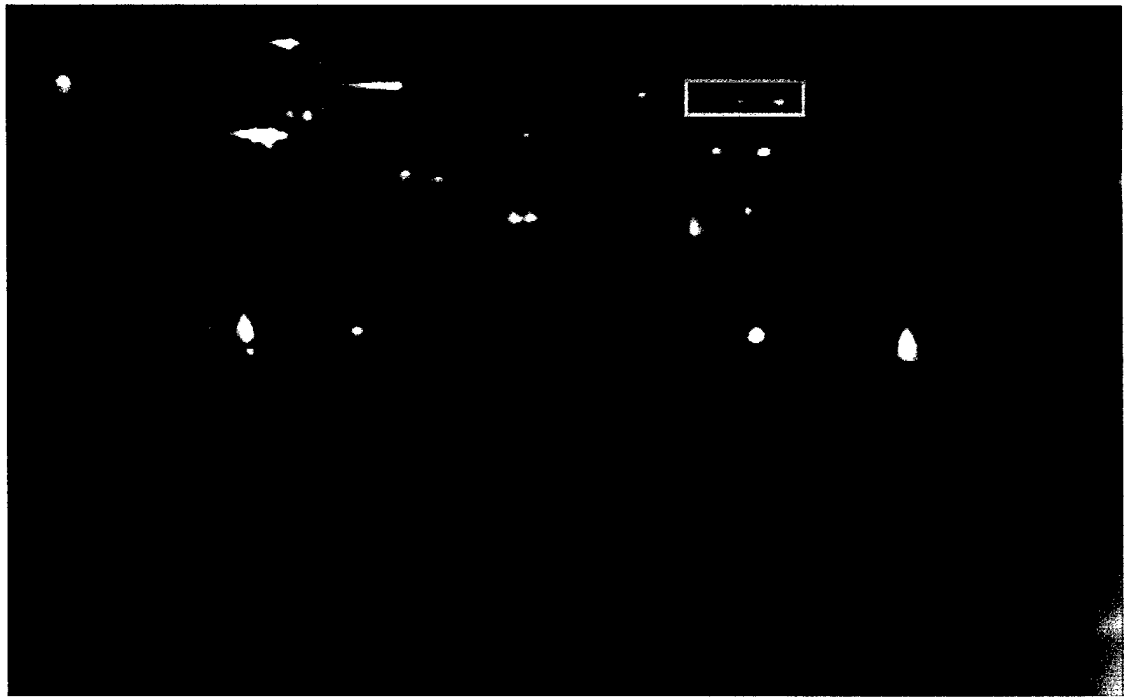


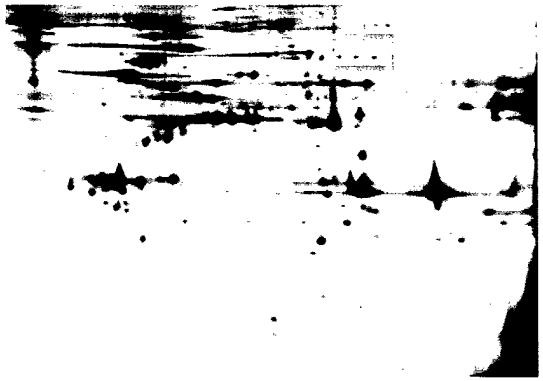
Figure 7. Comparison of pancreatic islet proteins from human organ donor HP159. Samples are prepared from pancreatic tissue (HP159O) and from purified pancreatic islets (HP159B). The left hand panel indicates the merged fluorescent images from Cy5 and Cy3 labeled samples while the right hand panels indicate the individual scans. The boxed region indicates the increased signal associated with 3 protein species upon islet purification while the yellow signals indicate proteins whose abundances are constant in the 2 samples.

DIGE 2D Gel Electrophoresis
Comparison of Human Islets During Purification

HP159O (Cy5) and HP159B (Cy3) DIGE 2D Gel



HP159O (Cy5)



HP159B (Cy3)

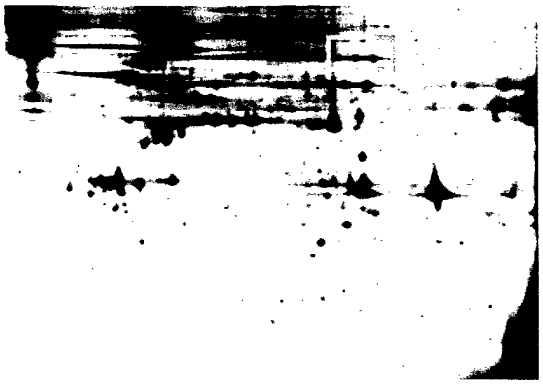


Figure 8. Statistical analysis of the 3 protein species that increase in signal when islets are purified. Symbols are: before purification, squares; after purification, circles; and mean signal form 5 islet donor isolations, crosses. The p-values were calculated using the paired t-test.

Paired T-Test of Donor Population Proteins During Islet Isolation

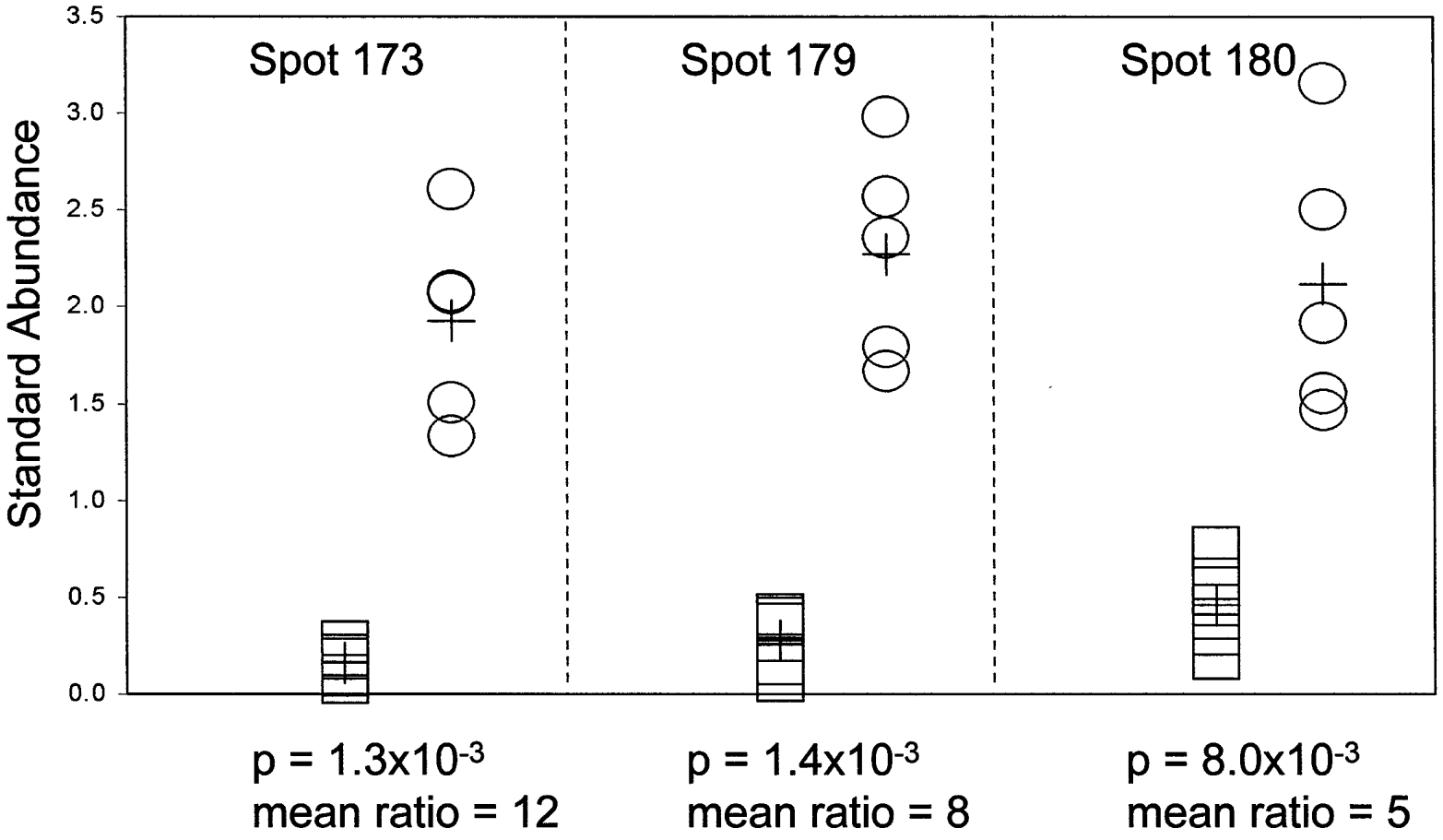


Figure 9. Mass spectra of protein spot 173. Analysis of the spectra indicated that the protein was bovine serum albumin a carrier protein added during islet preparation in order to enhance islet yield and function.

Mass Spectra Spot 173

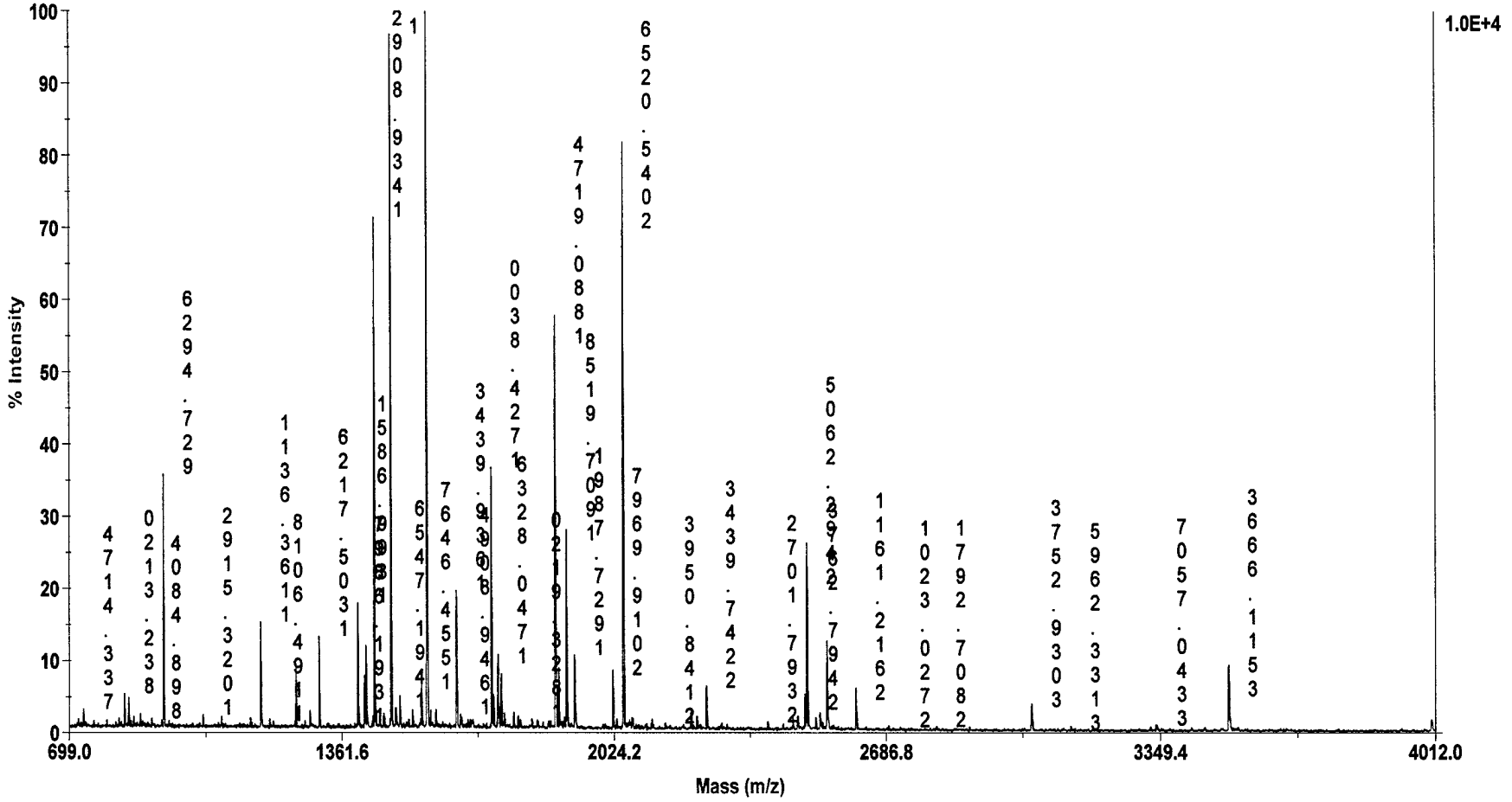
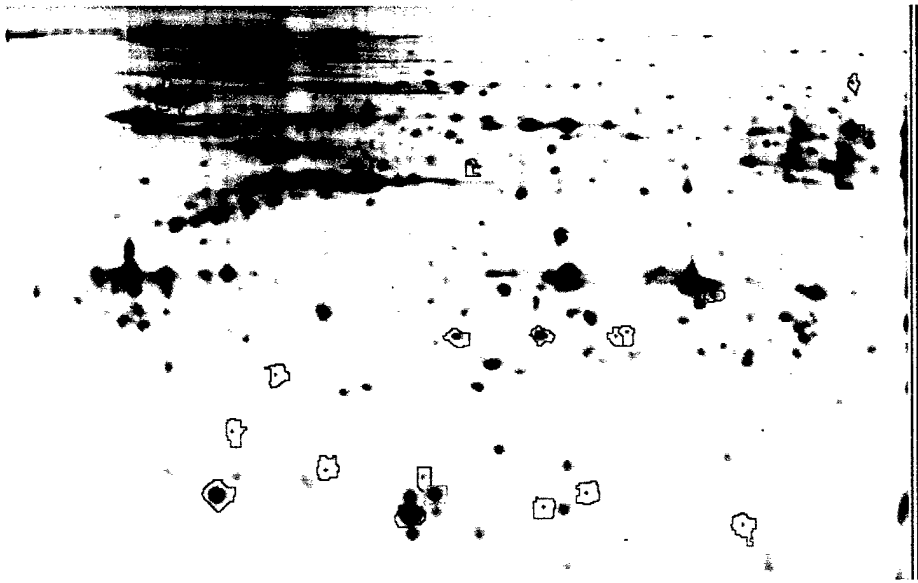


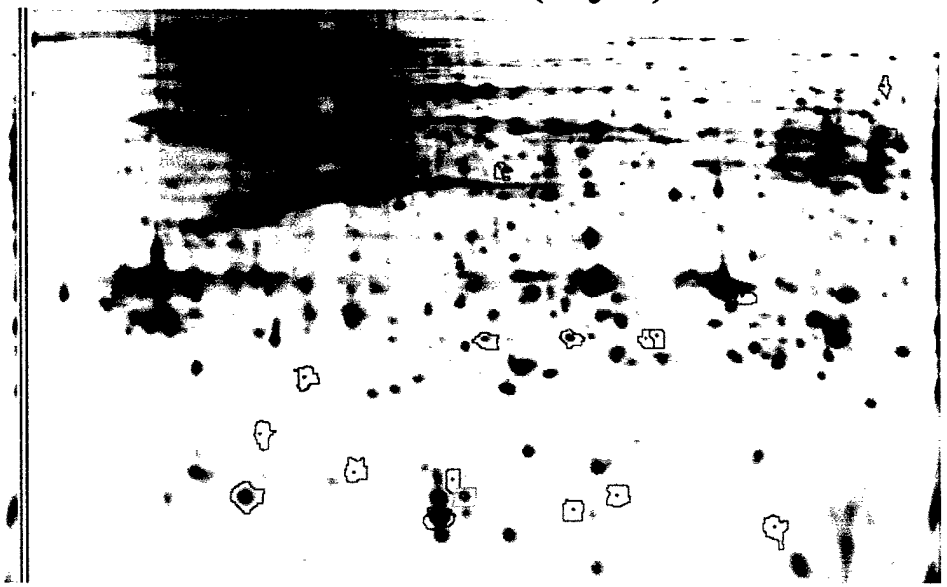
Figure 10. Proteomic analysis of islets purified from human organ donors HP162 (left panel) and HP161 (right panel). Of the 1114 proteins identified from the fluorescent scan greater than 98% exhibited identical abundance. The position of the less than 2% of proteins whose abundance is variable are indicated by circles in the 2D gel image.

Comparison of Cultured Islets HP161B and HP162B

HP162B cultured (Cy3)



HP161B cultured (Cy5)



Difference In-Gel Analysis

	Events	%
Decrease	19	1.7
Similar	1094	98.2
Increase	1	0.1
Total	1114	

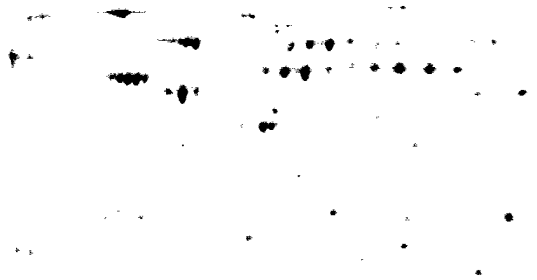
Figure 11. Comparison of protein isolated from total cellular and purified islet insulin granule fractions. The example uses proteins obtained from the rat-derived islet cell line INS1 and is a preliminary study to establish protocols for examining human islet granules. The left hand panel indicates the merged fluorescent images from Cy5 (red) and Cy3 (green) labeled samples while the right hand panels indicate the individual scans. The absence of yellow signals indicate that granule purification was successful.

Comparison of Granules from Rat Cell Line During Purification

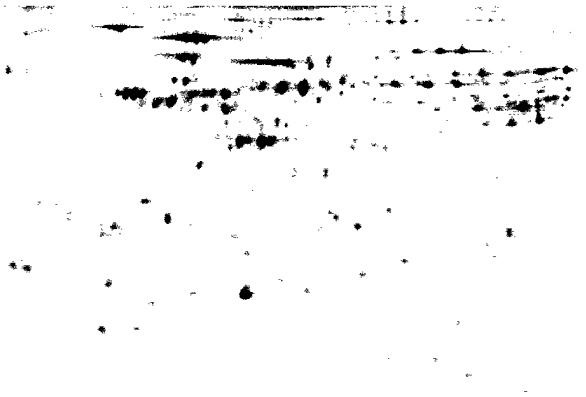
INS1 Cell Line



Before Purification (Cy3)



After Purification (Cy5)



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APPENDIX 2

GIFT-D ADDITIONAL MATERIAL

* Protocol
* Transcripts of two Education Modules for the Health Care Professional
* Abstracts and Manuscripts
* Assent forms
* Letter from University of Michigan

PROTOCOL

Protocol Title: *Genetic Information for Testing Diabetes (GIFT-D)* the program component of the *New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes:* Pilot Study and Phase 1

Justification for Proposed Study

Molecular technology developed by Dr. Trucco, with previous funding from the Department of Defense, is now available to test individuals in the general population and estimate their risk of developing genetically linked diseases, such as type 1 diabetes mellitus (T1D). At present, several research groups in the US are using genetic screening to identify newborns who carry T1D susceptibility genes for natural history studies or clinical trials. This presents educational and information-dissemination challenges, such as the accurate delivery of information regarding genetic risk. Genetic testing also has the possibility of leading to significant distress in some family members with the magnitude of this distress varying as a function of factors such as test results, method of risk notification (e.g., genetic risk education /counseling), coping resources, perceived risk, optimism, health beliefs, and pre-existing depression/anxiety. Efforts must be taken to meet these challenges and minimize distress.

Therefore, the major goals of this study are to develop, implement, and evaluate a genetic educational/ counseling program targeted at T1D families that, if effective, could be applicable to the general population. (The purpose of this program is to enhance the understanding of genetic testing for T1D; thus, enabling the families to make a more informed decision regarding whether or not to receive the test.) While the long-term goal of the project is to implement the program in a general population, this current feasibility study will be conducted utilizing siblings of children with diabetes who are seen at the Children's Hospital of Pittsburgh Diabetes Center. *(This feasibility study will be referred to as Phase 1.)* These siblings and their parents have a pre-existing familiarity with the disease. Prior to obtaining genetic testing to determine the child's risk of developing T1D, children and their parent(s) will receive a web-based genetic educational/counseling program. Program effectiveness will be examined by changes in knowledge, health beliefs (e.g., risk perception), attitudes (e.g., perceived stigma), and behaviors (e.g., monitoring vigilance). Base-line variables along with demographic and personality characteristics, and life events will be examined in regard to their effects on the outcomes of interest.

Additionally, to minimize distress due to genetic testing results, further counseling support will be provided to the child and parent(s) as they receive face-to-face notification of the actual risk status by a trained health care professional. Follow-up contacts with the family will also identify any individuals who may benefit from additional counseling. Children, who are found to be at risk for T1D, will be subsequently invited to join other ongoing screening studies (e.g., Trialnet).

The Children's Study at CHP is significant because it will: 1) develop, implement, and evaluate the effectiveness of an interactive internet-based educational/counseling program (for risk notification and genetic counseling) that will communicate information about genetics and T1D risk status to Health Care Professionals (HCP's), parents and children. Three separate programs (HCP, parent and child) will be specifically developed for the Internet; 2) evaluate the psychosocial, and behavioral impact (outcomes) of receiving T1D risk information and notification of T1D risk status; and 3) serve as a feasibility study for Phase 2 (general population clinical trial) which will be conducted on military bases on military dependants and their parents without T1D.

This proposed study will be conducted in two stages:

Stage One: Young Adult Pilot Study

A. Part One (assessment and education)

B. Part Two (testing and counseling)

Stage Two: Children's Hospital of Pittsburgh Diabetes Center (Phase 1)

A. Part One (assessment and education)

B. Part Two (testing and counseling)

In response to comments on a previous review by the HRC, we are now proposing to do a Young Adult Pilot Study. The committee previously raised the issue that it may be unethical to subject children to a study where the psychological risks are unknown. However, genetic screening, without genetic education and counseling, is currently being conducted to identify children at high risk for T1D for recruitment into natural history studies and clinical trials. We, therefore, believe that any potential familial distress related to genetic screening must be identified so appropriate interventions can be made.

It is for that reason, and on the direct advice from Dr. Ev Vogeley, that we now have incorporated a Young Adult Pilot Study. Prior to the implementation of the pediatric study, we will review the results of the Young Adult Pilot Study with Dr. Vogeley and other members of the HRC (if necessary), and if no evidence of psychological disturbance is found, we will ask the HRC to proceed with the rest of the study.

The Young Adult Pilot Study in Stage One will be conducted with 20 young adult males and females, between the ages of 18-25 yrs old, who have a sibling with T1D. The major purpose of this pilot study is to identify the potential for adverse psychological reactions (i.e., significant clinical scores on the depression and/or anxiety scales) in an adult population receiving information regarding one's own risk of DM. Predicated on both the successful completion and results of the first ten subjects of the Young Adult Pilot Study, we will proceed with conducting the Children's Study at CHP. The Young Adult Pilot Study will be discussed in its entirety under the section titled "Pilot Study".

This proposal represents The Young Adult Pilot Study and the Children's Study at CHP Phase 1. These studies have several consent forms, for the young adult, parent – child dyad, HCP, and a separate consent for the proband. They are: Consent to Contact (2), Consent to Contact a Collateral Informant (1), Consent for "Assessment and Education" (2), Consent for "Testing and Counseling" (2), Consent for HCP, and Consent for Proband (2).

Research Questions and Specific Aims (for the Children's Study at CHP)

1) To determine process evaluation of the internet-based educational/counseling program.
    a) How satisfied were the participants with the content and delivery of the program? How much time and effort did the program demand? Did it meet expectations?
    b) Did the program significantly increase the participant's [Health Care Professional (HCP), parent and child] level of knowledge of genetics and diabetes?
2) To determine outcome evaluation of the internet-based educational/counseling program.
    a) What is the effect of the education/counseling intervention on psychosocial outcomes (depression, anxiety, perceived risk, health beliefs, quality of life, and stigma) and behavioral outcomes (prevention behavior and disclosure) on child and parent?
    b) What is the effect of receiving notification of T1D risk status on psychosocial outcomes (depression, anxiety, perceived risk, health beliefs, quality of life, and stigma) and behavioral outcomes (prevention behavior and disclosure) on child and parent?
    c) Did the program change the individuals' perceptions of T1D risk?
3) To identify characteristics of subjects who agree to receive the genetic testing.
    a) What percentage of the subjects who received the education intervention had the genetic test?
    b) What percentage of the subjects who received the genetic testing had the counseling?
    c) Which psychosocial factors (depression, anxiety, perceived risk, health beliefs, quality of life, stigma, optimism, religiosity, and family function) demographics, and cognitive factors (knowledge) are associated with subjects' receiving genetic testing for T1D?

Pilot Study

Young Adult Pilot Study

A. Part One (assessment and education)

B. Part Two (testing and counseling)

Justification

In accordance with the Belmont Report, preceding the study in a sample of children from CHP, a pilot study will be conducted with 20 young adult males and females, between the ages of 18-25 yrs old, who have a sibling with type 1diabetes. The major purpose of this pilot study is to identify the potential for adverse psychological reactions (i.e., significant clinical scores on the depression and/or anxiety scales) in an adult population receiving information regarding one's own risk of DM. Previous studies examine psychological distress in diabetic adolescents and adults have used T-scores $\geq$ 65 as indicative of clinically significant impairment (Diabetes Control and Complications Trial Research

Group: Influence of intensive diabetes treatment on quality-of-life outcomes in the Diabetes Control and Complications Trial. *Diabetes Care* 19:195-203, 1996). We will use that same strategy in this study; a T score ≥ 65 is a score that is 1.5 standard deviation units higher than the mean for that study population.

Hypothesis

Out of 20 subjects, there will be no adverse reactions to receiving information regarding one's own risk of DM.

Subjects

Recruitment: Subjects will be recruited from the general population by means of advertisements placed in the local college newspapers and the *City Paper*, personal contacts of the researchers, and flyers posted at various campus locations. If interested, the advertisement will direct potential subjects to call the project office. (A "Waiver of Documentation of Consent" for this phone screening is being requested.) During this call, the potential subjects will be screened utilizing the GIFT-D Phone Screening Form (see attached) to insure they meet the eligibility criteria. Eligible candidates will be scheduled to come in for the first pre-education visit in Diabetes Institute, Suite 300 of the Keystone Building, 3520 Fifth Avenue.

Eligibility criteria: (1) males or females between the ages of 18 and 25; (2) have no diagnosis of type 1 diabetes; (3) have a brother or sister with type 1 diabetes; (4) no significant clinical score of depression or anxiety at baseline (significant scores will be referred to the clinician).

Termination: Subjects may be terminated for three failed appointments.

Sample Size: A total of 20 young adult subjects will be enrolled into this pilot study to collect preliminary information on the safety of the intervention that includes genetic testing related to type 1 diabetes. This sample size was not determined in order to have a certain level of statistical power when hypothesis testing but rather to explore the possible adverse psychological reactions in an young adult population receiving information regarding one's own risk of DM. However, with 20 subjects we anticipated that this sample size is feasible to enroll during a one month period of the time allocated for subject accrual.

Methods

This pilot study will be a one-group quasi-experimental, repeated measures design. It will entail 2 visits and one phone follow-up. The first visit will consist of a pre- and post-assessment and education intervention; the second visit (approximately 1-2 weeks later) will be a pre- and post-assessment and counseling intervention; and there will be a one-month follow-up phone assessment. The longest assessment period will take approximately 20 min. The educational/ counseling sessions will take less than one hour. The subjects will be reimbursed $50.00 at each visit to cover travel expenses and parking. They will also be compensated $10.00 for their time with the phone assessment.

Procedure:

At the first visit, in a private room, the Project Director or her Graduate Student Researcher will personally discuss the study with the subject, verify his/her eligibility, and explain the informed consent process. Using the "Assessment and Education" consent form, the coordinator will read the statement to the subject telling them that they have no obligation to participate in the study. The subject will then be given time to review the form before making a decision to participate. He/She will be asked if they have any questions or concerns that need addressing before they sign the consent. After the subject signs the "Assessment and Education" consent form, they will be given a copy of the consent form to take home which includes the name of the contact Co-PI (D. C-P) along with her phone number in case the subject has questions after he/she leaves. After the consent process is completed and a signature obtained, the baseline pre-education program assessment (see Table 4) will be administered. This battery of questionnaires includes measures of depression and anxiety. All assessments (except "evaluation survey" and "life events") are self-administered paper-and-pencil format. Subjects who have a T score of 65 or greater will meet threshold for a potentially clinically significant depression and/or anxiety disorder at baseline, and will be given a referral to a mental health provider and excluded from continuing in the study. They will be given their exit check for $50. In the event that subjects actually endorse items indicative of suicidality risk (e.g., on the Child Depression Inventory, endorse either 'I think about killing myself but would not do it' or 'I want to kill myself'), this information will be immediately forwarded to our project clinician (Dr. Tad Gorske; a licensed clinical psychologist) who will speak with the subject further to determine whether an immediate referral to a local mental health facility is indicated. Subjects without acute psychological distress will then view the genetic and diabetes educational program (Child/ Adolescent version) on the computer, after which, they will complete the post-education program assessment (Table 4). It was decided that the young adults would view the adolescent version since this version has been developed in the first person and directly refers to the individual potentially being tested. The alternative parent application is designed specifically for parents to make a decision for their child. Subjects will then be asked whether or not they wish to provide a cheek cell sample for the genetic testing. Subjects who refuse, will be given the 'Why Not Test" questionnaire exploring reasons to discontinue the project. Subjects who agree to provide the sample, will sign the second consent "Testing and Counseling". The cheek cell sample will be collected (by the Project Director or her Graduate Student Researcher. Samples will be hand labeled with the participant's study number and the date. Samples will be hand delivered to the Genetics Lab in the Rangos Building. After testing, samples will be destroyed) and the subject scheduled for their second visit. All subjects will be given $50 at the end of this visit regardless of their level of participation.

At the second visit, approximately 1-2 weeks later, subjects will complete the pre-counseling assessment (Table 4). They will view a genetic counseling program

(Child/Adolescent version) on the computer. Subjects will decide whether or not to receive the results of their personal risk. Subjects who refuse will be given the 'Why Not Counsel" questionnaire exploring reasons for not wanting to receive the results of the test. Subjects who agree will meet face-to-face with a Health Care Professional who will explain their personal risk and answer questions. (This Health Care Professional will have participated in the Health Care Professional genetic education module. They will also be personally trained by Ms. Betsy Gettig, a certified genetic counselor, regarding the appropriate strategies for communicating genetic risk information to relatives of individuals with type 1 diabetes.) At the conclusion of this counseling session, another assessment will be completed, including measures of depression and anxiety. Subjects who have a T score of 65 or greater and thus meet threshold for a potentially clinically significant current depression and/or anxiety disorder will be given a referral to a mental health provider for further evaluation. As part of the referral process, subjects will discuss their feelings with our project clinician, Dr. Tad Gorske, who will not only evaluate their level of psychological distress clinically, but will get information about events associated with this distress. This will allow us to determine the extent to which their depression is related to study procedures, or to other life events (e.g., death in the family). Referrals to mental health providers will be provided if warranted. All subjects will be given $50 at the end of this visit regardless of their level of participation.

At one month, over the phone, subjects will be contacted to complete the depression, anxiety, and monitoring vigilance measures. Subjects who have a T score of 65 or greater will meet threshold for a potentially clinically significant depression and/or anxiety disorder will be interviewed by our project clinician, Dr. Tad Gorske, and will be given a referral to a mental health provider for further evaluation, as described above. All subjects will receive a check for $10 in the mail for completing these measures.

Measures

The young adult assessments are modified versions of the parent questionnaires. For a description of the instruments, scales, score interpretation, and psychometric properties, see Measures section of the Children's Study at CHP protocol in this proposal.

Data Analyses and Interpretation

Appropriate descriptive statistics will be computed to characterize the safety profile with respect to depression and anxiety. Continuous type variables (e.g., anxiety, depression) will be summarized using means, standard deviations, and ranges, while categorical variables (e.g., dichotomized depression variables based on clinically meaning cutpoints) will be described using frequency counts and percentages. Change scores relative to baseline values will be computed to describe changes in psychological profile over time as subjects proceed through the study. Small sample confidence intervals will be computed for means and percentages, especially with respect to depression and anxiety.

Predicated on the successful results of the first ten subjects in this adult pilot study (i.e., the first 10 subjects will have no adverse reactions to receiving information regarding one's own risk of DM), we will proceed with conducting the Children's Study at CHP. If any type of distress is identified, the protocol will be modified accordingly and re-submitted for IRB review.

Research Design

Children's Study at Children's Hospital of Pittsburgh Diabetes Center (Phase 1)

A. Part One (assessment and education)
B. Part Two (testing and counseling)

The project entails a one-group quasi-experimental, longitudinal, repeated-measures design, embedded within an educational/ counseling intervention prior to and following genetic testing for type 1 diabetes. Subjects are evaluated pre- and post- education, pre- and post- counseling; and at a one-month and three-year home follow-up.

Subjects of the Study

The sample will be parent – child dyad obtained from the CHP Diabetes Center utilizing the following inclusion/exclusion criteria:
The child must have a full biological sibling less than 21 years of age with T1D (proband), be between the ages of 7 and 18 yrs., be in a home with at least one biological parent who is willing to participate, and be able to speak and read English. Neither the parent or child can have a significant clinical score of depression or anxiety at baseline (significant scores will be referred to the clinician). Subject must not be a Trialnet participant nor be adopted.

Sample Size Justification

We estimate approximately 250 siblings to be eligible for this Phase 1 study. However, for this exploratory study, we plan to recruit a feasible sample size of 50 families having a T1D child as a proband. Although this sample size was not determined on the basis of achieving specified level of statistical power, we would have sufficient power (.80) to detect time effects as small as f=.286 for 2 assessments and f=.258 for 3 assessments in time at a two-tailed significance level of .05.

Given the longitudinal design of the study, subject attrition is possible. To insure 50 families complete the program evaluation, 70 families will be enrolled, conservatively adjusting for about 28% attrition.

Recruitment Methods

Following the successful completion of the Young Adult Pilot Study, brochures (attached) will be distributed at the CHP Diabetes Center. These brochures contain a description of our research project for risk of T1D, eligibility criteria and contact information.

In Phase 1, siblings from families with one child already diagnosed with T1D will be recruited from the CHP Diabetes Center. Families will be recruited during a regularly scheduled clinic visit of the child with T1D (proband). During this visit, a health care professional (HCP) will ask the family if they are interested in talking to the study coordinator about our study. If they are interested, the HCP will have the parent sign the

Consent to Contact and introduce them to the study coordinator. Should the proband be 18 yrs old or above, he/she will sign a Consent to Contact specifically designed for him/her. In a private clinic room, this coordinator will personally discuss the study with the family, determine their eligibility using non-identifiable characteristics and explain the informed consent (assent) to those families who are eligible and interested. The coordinator will ask if they have any questions or concerns. The name of the contact Co-PI (D. C-P) along with her phone number will be provided. Using the consent form, the coordinator will read the statement to the family telling them that they have no obligation to participate in the study and their care will not be affected by their decision. A copy of the consent will be given to them to take home to review before signing. We will ask that both parents sign the consent form. The proband will also be recruited to provide a sample for DNA testing at the next visit. This sample will be used to help determine the sibling's risk. An appointment will then be scheduled (separate from the regular diabetes clinic visit) where the parent and all eligible sibling/s (ages 7-18) and the proband will begin their participation in the study.

Procedure After Recruitment

At the 2nd visit, , the participating parent and all eligible siblings will be asked to sign three copies of the Consent/Assent form(s). Contact sheets and the Consent to Contact a Collateral Informant (see after 3 year follow up) will be completed and a study number assigned. A unique ID and password (basic authentication scheme) will be issued for parent and each child to restrict access to the website. Families will be given instructions on the use of the Internet-based application. They will complete the web-based baseline psychosocial assessments and the genetic pre-test questionnaire (knowledge, beliefs, etc.). Demographic characteristics (e.g., age, sex, race, ethnicity) and family history of diabetes will also be assessed. Some of this information will be included in the risk algorithm so that an estimate of T1D risk can be determined. The parent and participating sibling(s) will separately complete the web-based genetic and diabetes educational components. (Subjects who have a T score of 65 or greater will meet threshold for a potentially clinically significant depression and/or anxiety disorder at baseline, and will be given a referral to a mental health provider and excluded from continuing in the study). The proband may also view the educational program if desired. The site coordinator will be available for assistance. Developmentally appropriate programs will be shown. Following the program, a short post-test web based assessment will be conducted. The family's experience with the web-based education modules will be discussed and logged for formative (process) program evaluation. Any questions they have regarding genetics, genetic testing for T1D, etc. will also be answered at this time. The family will be again reminded about the validity and utility of genetic tests for T1D, and the risks and benefits associated with being tested. All participants will have the opportunity to decide if they wish to proceed with the genetic testing. If they decide not to continue, they will complete a short questionnaire exploring their reasons for not wanting to have the testing done, along with an exit survey. This visit will take approximately 2 hours.

For those who agree to the testing, the Consent/Assent for Genetic Testing and Counseling will be explained and signatures obtained. Additional consents will be explained and signatures obtained for the proband sample collection. (There are two

consents, one for a proband under 18 and one for a proband over 18). Cheek cells will be collected using the mouthwash technique or soft brush from each assenting child and the proband. (The web based computer program will generate labels and a packing slip. The subject' study number and date will appear on the label to be placed on the sample. Samples and the packing slip will be sent to the genetics Lab in prepaid mailing containers for testing. After testing is completed, the sample will be destroyed.) A third visit will be scheduled for the parent and sibling/s to return for the risk notification and counseling component. Each participant (parent, siblings and proband) in this 2nd visit will receive $25.

During Visit #3, scheduled approximately two to three weeks after Visit 2, the parent will complete a Monitoring Vigilance questionnaire. All participants will complete a developmentally appropriate web-based genetic counseling session. At the end of this session, participants will decide whether they will receive their risk status notification and post-test genetic counseling. Those deciding not to proceed will complete a questionnaire exploring their reasons for not continuing and an exit survey. Those deciding to proceed will meet the HCP to receive their risk estimate. The HCP will meet with each participating sibling and parent and while together, each child will be given his/her personal risk assessment. Personalized risk estimates have been calculated using the risk algorithm (Research Question 1) and the results of the mouthwash test (i.e., DQA1-DQB1 haplotypes), as well as family history and demographic information obtained during Visit #2. In addition to the information they receive from the trained HCP, participants will receive a written summary of the genetic information provided. Families will also receive information about possible T1D research and intervention studies. Parents and children will then complete a brief psychosocial assessment. All participants will be given $25 for this visit. Home computer access will be determined in preparation of the next data collection point. Those families without access to a computer can complete their follow up in the clinic. This visit will take approximately 2 hours.

One month later, each family will be contacted to complete a short battery of web-based questionnaires online. These will include: Psychological Status, Stigma/Discrimination, Behavior Changes and an Exit Survey. This battery will take approximately 15 minutes. A check for $25 will be mailed to all participants completing these questionnaires.

In addition, after 3 years, each family will be contacted via telephone, letter or postcard to request that they complete an online battery of questionnaires as a follow-up to the study. (The Consent to Contact a Collateral Informant was signed during the second visit to allow us to contact other family members/friends in the event we are unable to reach participant families for follow-up.)

Termination

Subjects may be terminated for three failed appointments

Study Personnel

The two study personnel that will interact in the clinic with the families are the Project Director and the site coordinator. The Project Director's role is to oversee day-to-

day activities of the entire program intervention component of the proposal, and will serve as project coordinator for the clinic site. Her duties include coordinating all aspects of this project, including training and directly overseeing of study staff and preparing the training materials for the training sessions. She will also be responsible for overseeing the recruitment of subjects, maintaining subject files, and assuring standard delivery of the study protocol. She will be the contact person for each of the site research nurses with respect to protocol issues and data management.

The site coordinator's will be responsible for recruiting the families at the clinic site that meet study criteria. She will also be responsible for providing potential subjects with a copy of the Consent, obtaining a signed Consent/Assent form, orienting families to the computer programs, providing face-to-face information and counseling. In addition, she will be setting-up the computer, overseeing the on-line data collection at the site, and overseeing the intervention in the clinic. She will also serve as a liaison for the clinic site, and maintain correspondence with the central School of Nursing study office.

Measurement

Table 1 lists the variables and measures with the number of items that will be used to collect the data. ***Table 2*** lists the data collection points of each measure to be collected for the child, and ***Table 3*** lists the data collection points of each measure for the parent. These measures evaluate the mediating or outcome effects of the education and counseling program. Children and parents will be asked to separately complete modules to assess knowledge, psychosocial parameters and behaviors, as outlined in Table 1. Each education module will be preceded by a short pre-test and followed by a short post-test that will take approximately ten minutes to complete. The Expanded Health Beliefs Model (EHBM) and models of Stress and Coping modified for T1D will guide the assessments. The EHBM hypothesizes that, in addition to knowledge about disease prevention, an individual is more likely to engage in a recommended health behavior if he or she feels susceptible to a health problem, perceives its complications to be serious, considers the recommended behavior(s) to be beneficial in maintaining health or preventing complications, and believes that the benefits of following the recommendations outweigh the barriers. Thus, the dimension of the EHBM will be operationalized as: 1) perceived susceptibility for developing type 1 diabetes, 2) perceived severity of the disease, 3) perceived benefit of receiving genetic testing and genetic counseling, 4) perceived barriers to having the genetic testing, receiving genetic counseling or being identified as an individual at 'high risk' for the disease, and 5) intention to have the genetic testing and counseling performed.

Well validated/reliable, and newly developed instruments will be modified for the web-based interactive application as follows:

The mature, well validated measures to be used are: the State-Trait Anxiety Inventory (STAI) and State-Trait Anxiety Inventory for Children (STAI-C) (Spielberger); Children's Depression Inventory (CDI-s) (Kovacs); Center for Epidemiologic Studies Depression Scale (CESD-10) (Radloff); Life Orientation Test for Optimism Revised (LOT-r) and Life Orientation Test for Optimism Pediatric version (LOT-P) (Scheier); Child Health Outcomes (Young-Hyman); Family APGAR (Smilkstein).

Well validated measures that have been modified include: Risk Perception Survey for Developing Diabetes (Attitudes about Health), (child and parent version) (Walker); Child Health Outcome (child version)(Young-Hyman); Diabetes Health Belief for Genetic Testing Questionnaire (child and parent version) (Wang); Quality of Life Health Ladder (Andrews and Withey); Perceived Stigma (child and parent version) (Berger); Satisfaction With Genetic Education (Castaldini); Satisfaction with Genetic Counseling (Castaldini and Shiloh); and Monitoring Vigilance (Baughcum and Johnson et. al.).

Although theoretically and empirically based, measures that have been developed for the purpose of this study are: Demographic Questionnaire; Religiosity; Genetic/Diabetes Knowledge Test (child and parent version); Intention; Self-Efficacy; Refusal (Exit Survey); Disclosure Questionnaire; Life Event Log; and the Process Evaluation logs of Offensive/Expectations, Time/Effort and Assistance.

Changes in prevention behavior will be determined by conducting a post-intervention assessment (Monitoring Vigilance) at 1 month and 3 years on the proportion of participants who receives genetic testing to assess whether they perform self-initiated lifestyle behavioral changes to minimize risk of developing diabetes (e.g., diet), prescribe to health behavior changes to minimize risk (e.g., enrolls in prevention trial), or seeks other information on the internet-based resource. In addition, we will examine the factors that may influence such intentions and behaviors, such as age, gender, perception of risk, etc. It is important to emphasize that behavior changes will not be viewed as an indication of the program's success. Rather, they represent observations of interest that may be further explored in an extended follow-up of this cohort. Subjects will be asked to complete measures that evaluate decision-making processes (i.e., Monitoring Vigilance and Disclosure). In addition, a process evaluation will be conducted to determine time/effort expended, subject satisfaction, identification of offensive information, and goal expectations. Psychometric analyses will be conducted on all measures to determine the validity and reliability for this population for both parent and child versions.

Table 1 Data to be collected

Knowledge
Diabetes
Complications (acute and long-term)
Genetics
Inheritance of type 1 diabetes
Environmental risks factors
Approaches for prevention
Psychosocial parameters
Perceived risk
Health beliefs/ intention
Anxiety
Depression
Optimism
Health quality of life
Stigma / discrimination (insurance, employment)
Religiosity
Family Function
Life Event Log
Behaviors
Receives genetic testing
Receives counseling
Prevention Behavior changes (health and/or lifestyle related)
(e.g., diet, seeks prevention tx) @ 1 month and 3 years
Seeks other information on the web-based resource
Disclosure
Process evaluation
Time/effort
Satisfaction
Offensive/expectations

Health Care Professionals Training

A second objective of our proposal is to increase the availability of genetic information to health care professionals, as well as improve their understanding of genetics so that they may provide this information to their patients who seek it.

We will identify one health professional from the CHP Diabetes Center to serve as the site coordinator. This individual will be responsible for recruiting families from his/her clinic. He/she will be oriented according to protocol by the study coordinator and will be provided with a procedure manual. He/she will receive a web-based training program, currently being developed, which will also be provided for any other clinic Health Care Professionals (HCP) interested in participating. Additionally, he/she will also be trained to provide the web-based genetic education/counseling program and assessments, conduct the genetic testing, and deliver both the face-to-face risk

notification and the web-based post-counseling program to the families that complete all components of the study. This HCP program will include training in basic genetics, genetic testing, genetic counseling, and ethical, legal and social issues related to genetic testing. HCPs will receive extensive information about type 1 and type 2 diabetes, and the acute and long-term complications associated with these diseases. The study coordinator and the co-investigators will be available to the HCP while he/she is functioning as the site coordinator for questions and support. He/she will also be updated regarding the status of the ongoing T1D intervention trials. In addition, HCP-client communication skills will be addressed. In this manner, HCPs will be able to fully participate in the risk notification and risk evaluation processes outlined in the current proposal.

Timetable for Completion of Each Phase of the Study: 11/01/02 to 10/31/04

During year 1: hiring and training personnel; purchasing equipment; finalizing and verifying questionnaires; developing three separate (one for parents, one for children, and one for health care professionals) web-based interactive educational/ counseling programs with CIDDE; developing questionnaires on-line, and an Internet data tracking program with DSI; generating data dictionaries; compiling manuals for intervention protocols; setting up study sites and orienting on-site health care professionals; recruiting conducting, and evaluating the *Young Adult Pilot Study* from 11/03 thru 2/04.

During year 2: recruiting and conducting the *Children's Study at Children's Hospital of Pittsburgh Diabetes Center (Phase 1)*; conducting the one-month telephone follow-ups on subjects, thus completing all the initial data collection; data management; conducting analyses; preparation and submission of final written reports; and begin planning preparations for program implementation of Phase 2 (general population clinical trial) which will be conducted on military bases in military dependants and their parents without T1D.

Data Analysis and Interpretation

Data entered on the web-based password-protected forms by the subjects will be captured and stored directly onto a secure web server. Data will be downloaded from the web server and analyses carried out on project computers.

Prior to conducting the major analyses, we will employ exploratory data analysis techniques to thoroughly describe each variable and, which will, in part, determine the actual analysis required to answer the stated objectives. When statistical assumptions are reasonably satisfied, the application of data transformations or non-parametric methods will be considered. In the event of missing data, multiple imputation techniques will be used. Those who do not complete the intervention will be analyzed separately to determine any differences from those who remained in the follow-up. However, dropouts will be included in the final analysis.

In regard to the risk algorithm, an algorithm based on data collected for the Familial Autoimmune and Diabetes Study (JS Dorman, PI) was developed early in Year 1. It was based on computationally intensive methods to obtain estimates of risk, as well as determine the error in such estimates. A table of group-specific absolute risks have

been generated according to age, family history of T1D diabetes, and the families' DQA1-DQB1 haplotypes. See appendix for the "Age Specific Probability Estimates" and "Covariate Patterns" tables

For research question #1a, appropriate descriptive statistics (e.g., means, medians, standard deviations, and interquartile range for continuous variables and frequency counts and percentages for categorical variables) will be used to characterize participant satisfaction with the content and delivery of the education/counseling program. Expectations of the program and demands on the subjects in terms of time and effort will also be quantified and appropriately summarized.

Repeated measures modeling methods (e.g., covariance pattern models using mixed modeling methods) will be applied to explore the effects of an education/counseling program and received notification of T1D risk status on knowledge (Research Question #1b) and psychosocial and behavioral outcomes (Research Questions #2a, #2b, #2c) over time. This analytic approach is fairly flexible, allowing for modeling of continuous, discrete, or count type response variables; unequally spacing repeated assessments; data that is missing at random; and fixed and time-varying covariates. Longitudinally measured psychosocial outcomes such as depression, anxiety, perceived risk, health beliefs, quality of life, and stigma; and prevention behaviors (behavioral outcomes) will be assessed longitudinally. The behavioral outcome of disclosure, assessed only at follow-up, will be examined using descriptive statistics. In addition, the relationships between longitudinal outcomes and potential covariates will be examined using the repeated measures modeling methods previously described. For the modeling of the behavioral outcome of disclosure as a function of covariates, logistic regression analysis will be used. Given the exploratory nature of this pilot study, emphasis will be placed on the estimation of effect sizes rather than on the testing of hypotheses.

Research questions #3a and #3b will be analyzed using appropriate descriptive statistics. Regarding Research Question #3c, appropriate correlational and multivariate analyses will be performed to determine a predictive model for receiving genetic testing.

Benefits and Risks

The primary benefit to subjects participating in this research project will be education about genetics and diabetes through an Internet-based application. This will enable them to identify the risks and benefits of genetic testing so they will be able to make an informed decision as to whether or not they want their child/ren to undergo genetic testing to determine each child's risk for developing type 1 diabetes. The primary benefit to participating in the second component of the research study will be to actually learn their child/ren's personal risk of developing type 1 diabetes. Information regarding T1D, family support, and research studies will also be provided. This will be done both face-to-face with a Health Care Professional and on an Internet-based application. There is no direct benefit to the proband for participating in this study.

The primary risk associated with this study is that subjects may experience some psychological distress at having to be told about the results and risk of getting diabetes. To help ease this distress, we have added both an Internet based and a "face-to-face" counseling session to discuss what this risk really means. We will also provide referrals for both psychological and genetic issues if warranted.

Subjects might feel uncomfortable sharing personal information on questionnaires. To reduce this discomfort, we are using standard questionnaires in the Internet-based application and subjects will always have the option to not answer specific questions.

Subjects may also experience some feeling of discrimination, possibly in obtaining employment or insurance due to their knowledge of this risk information. However, since only the subject and study personnel know this information, this risk is minimal.

There is also a risk that unauthorized individuals might gain access to information contained within the website. However, since the site is user ID and password protected and only study number identifies the information contained therein, this too is a minimal risk.

The benefit for the HCP may be learning more information about type 1 diabetes and genetics. There are no risk associated with their participation.

"Unanticipated problems involving risk to subjects or others, serious adverse events related to participation in the study and all subject deaths should be promptly reported by phone (301-619-2165), by email (hsrrb@det.amedd.army.mil), or by facsimile (301-619-7803) to the Army Surgeon General's Human Subjects Research Review Board (HSRRB). A complete written report should follow the initial telephone call. In addition to the methods above, the complete report can be sent to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-ZB-QH, 504 Scott Street, Fort Detrick, Maryland 21702-5012"

Subject Protection and Privacy

In order to protect the safety of the subjects in the study, the depression survey for all subjects will be scored by the system in real-time. If the score is at or above the established threshold, the site coordinator and the study coordinator will immediately be notified by pager and e-mail. The site coordinator will ensure that the patient is safe and if necessary, a referral will be made to a local mental health practitioner.

In compliance with the Health Insurance Portability and Accountability Act (HIPAA), none of the subject's private health care information will be used for the research purposes of this study without his/her consent. All parents will be informed of the specific uses and disclosures of their child's medical information for the purpose of this research study and who will have access to that health information. This research study will not involve the recording of existing medical information nor will any medical information that becomes available while the subjects are participating in this study be placed in their child's hospital and/or physician records. If the results of this study are published, information concerning their child/ren will be in a form such that s/he cannot be identified. The subject's participation in this study will **not** result in health information being placed in the Children's Hospital of Pittsburgh medical chart, outpatient chart, or research record. All Research records will be kept confidential. Paper records will be stored in locked cabinets. Representatives of the U.S. Army Medical Research and Materiel Command (USAMRMC) may inspect the study records.

The study coordinator will be responsible for creating a unique ID for each participant in the project. Data collection and storage will be based on the unique study ID. No patient identifying information will be stored in the database. The key linking the subject identity with the study ID will be stored in a double locked file cabinet by the study coordinator.

Study participants will access the website and answer questionnaires through the World Wide Web Secure Socket Layer encryption will be used to provide full 128-bit data encryption. Project staff will access the site through a thick Java-client application. All data transactions between the thick client and the database will be encrypted using the encryption module of Oracle 9i.

Data Safety Monitoring Plan

The Principal Investigator will be responsible for data and safety monitoring for this project. Regular reviews of the accrued research data and other relevant information will be conducted to ensure the validity and integrity of the data. In addition study procedures will be reviewed to ensure that the privacy of research subjects and the confidentiality of their research data have not been violated. The table below outlines the elements to be reviewed.

Element to be Monitored	Method	Frequency
Data quality and integrity	Review of system audit information	Monthly
Study progress	Review of system generated subject tracking reports	Monthly
Depression survey notification	Review of system generated reports of depression risk notification	Monthly
Adverse event data	Manual review of adverse event reports	Monthly

Summary results of monitoring activities will be reported to the HRC upon request for renewal and will include the following:

- Frequency of monitoring that occurred during the study period
- Summary of cumulative adverse event data
- Conclusions related to change in risk benefit

The P.I. will report to the IRB within 24 hrs. any serious adverse event occurring at CHP that is associated with this research intervention. Unexpected adverse reactions of moderate severity occurring at CHP in association with the research intervention will be reported to the IRB within 5 days. Sponsor-generated safety reports will be submitted to the IRB with 30 days of their receipt by the investigators. Reporting of adverse events to sponsors and federal agencies will be the responsibility of the P.I.

Any departure/deviation from or modification to the protocol will be submitted to the University of Pittsburgh Institutional Review Board and the Army Surgeon General's Human Subjects Research Review Board.

Qualifications of the Investigators

Dr. Dorothy J. Becker, is a Professor of Pediatrics, Director, Division of Endocrinology, Metabolism and Diabetes Mellitus in the Department of Pediatrics at the Children's Hospital of Pittsburgh, University of Pittsburgh, School of Medicine. Dr. Becker received her M.B.B.Ch. degree from the University of Witwaterstrand, Johannesburg, South Africa and arrived here in 1974 to begin her Fellowship in Endocrinology at the Children's Hospital of Pittsburgh, Pennsylvania.

Initially interested in nutrition and hormones, Dr. Becker contributed to the medical community's understanding of complications associated with diabetes in childhood; she's also pursuing ways to predict the onset of the disease in those at risk. She was recently awarded a NIH grant, a multi-center intervention study comparing standard vs hydrolyzed formulas as a weaning diet in infants with genetic risk for developing type 1 diabetes. Dr. Becker has been an invited lecturer, symposium speaker, and chairperson at numerous international congresses and symposia.

Dr. Denise Charron-Prochownik, Associate Professor School of Nursing and the Graduate School of Public Health at the University of Pittsburgh has both provided twenty years of service and has had an active program of research for thirteen years in diabetes. Her work has included national diabetes activities, such as editorial board member and associate editor of Diabetes Care, editorial board member of the Diabetes Spectrum, and selected as a member of both the Health Care and Education Advisory Group of the National ADA Executive Committee and the ADA's 2002 Scientific Session Meeting Committee. She is a pediatric diabetes clinical nurse specialist and a pediatric nurse practitioner. Her research emphasis is in theory-based studies identifying cognitive/ psychosocial predictors of health behavior change in children and adolescents with diabetes. She has conducted several funded projects in the area of instrumentation, program development and evaluation, and survey designs.

Dr. Janice S. Dorman, Professor of Epidemiology, Associate Dean for Research, Graduate School of Public Health, University of Pittsburgh, has research interests which include the molecular epidemiology of autoimmune disorders, and their impact on women's health. She teaches an introductory and a laboratory-based course in molecular epidemiology. She also co-directs the WHO Collaborating Center for Diabetes Registries, Research and Training.

Dr. Dorman has served as Principal Investigator of grants investigating: 1) the molecular epidemiology of type 1 diabetes diseases worldwide, 2) the familial clustering of autoimmune diseases, and 3) the menopause transition among women with type 1 diabetes.

Dr. Christopher Ryan is Professor of Psychiatry, Psychology, and Health & Community Systems, and is a Vice-Chair at the University of Pittsburgh Institutional Review Board. His research career has focused on the effects of medical disorders (like diabetes) on psychological and neuropsychological function, and he has recently completed several large scale community-based intervention studies.

Dr. Linda Siminerio is currently the Director of the Diabetes Institute at the University of Pittsburg and she has coordinated the pediatric diabetes program at Children's Hospital of Pittsburgh since 1976. She has been involved with pediatric diabetes research and is the author of numerous scientific and consumer publications on diabetes. She is the co-author of the American Diabetes Association's (ADA) guide for pediatric diabetes care Raising a Child with Diabetes and book Goals for Diabetes Education. She has served as an associate editor of Diabetes Care, Pediatric Diabetes, editor of the clinical and education journal Diabetes Spectrum and is the current editor-in-chief of Diabetes Forecast.

Currently, Dr. Siminerio has been involved in global efforts to promote diabetes education, nutrition and the psychosocial aspects of diabetes. With her work with the International Diabetes Federation (IDF), she has served as Chair of the Education and Behavioral Aspects of Diabetes Satellite Symposia for the past three IDF Congresses hosted in the U.S., Japan and Finland. Most recently, she chaired the first IDF Congress track dedicated to education and the psychosocial aspects of diabetes in Mexico. She has been an appointed member of the IDF Section on Diabetes Education and the Board of Management. At the IDF Congress in Mexico in November, 2000 she was elected as one of 12 Vice Presidents serving the international diabetes community

Dr. Massimo Trucco, Professor of Pediatrics, is the Principal Investigator of the DoD application. He conceptualized and wrote this application after having secured the collaboration of scientists, clinicians, and computer experts at the Children's Hospital of Pittsburgh, University of Pittsburgh, and the University of Pennsylvania.

Dr. Trucco's interest in the prevention and prediction of Type 1 diabetes, together with his goal of making pancreatic islet transplantation a suitable therapy for young diabetic patients attracted the attention of the community in Pittsburgh and, in particular, of administrators at Children's Hospital of Pittsburgh and at the University of Pittsburgh. On this basis, the idea of creating a new Diabetes Institute in Pittsburgh was not only formulated, but quickly implemented. Dr. Trucco, also serves as the Director of the Juvenile Diabetes Research Foundation (JDRF) Center for Gene Therapy Approaches to Type 1 Diabetes.

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ANNUAL REPORT

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APPENDIX 2:

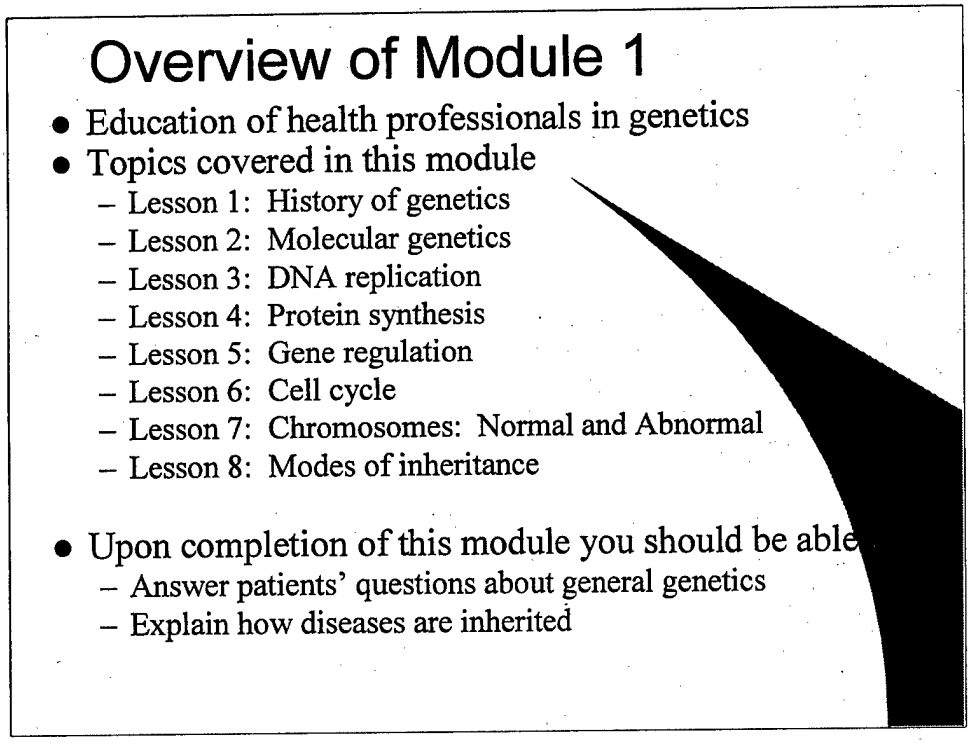
GIFT-D ADDITIONAL MATERIAL

TRANSCRIPTS OF TWO EDUCATION MODULES FOR THE HEALTH CARE PROFESSIONAL

Genetic Education for Health Professionals

General Genetics





It is becoming increasingly important for health professionals to have a working knowledge of general genetics. Although much information is available, educational opportunities are limited. To address this need, we have developed a series of three modules using power point slides. Module 1 is about general genetics, module 2 is about the genetics of diabetes, and module 3 is about genetic counseling.

This is the first module; it covers the history of genetics, molecular genetics, DNA replication, protein synthesis, gene regulation, the cell cycle, chromosomes (normal and abnormal), and modes of inheritance.

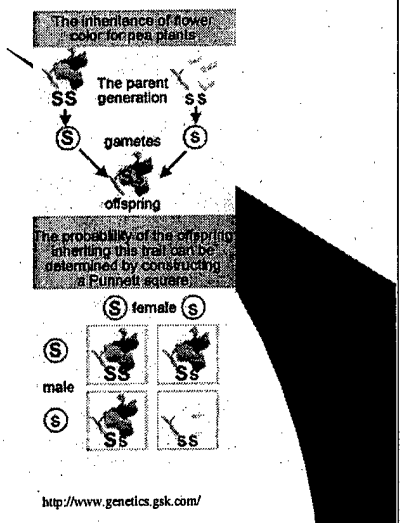
Upon completion of this module, you should be able to answer patients' questions about general genetics, and explain how diseases are inherited.

Lesson 1 – History of Genetics

- In lesson 1, the history of genetics will be discussed. The evolution of the science of genetics is information anyone working in the health professions should know, as it impacts how clinical research is conducted and has important implications for the advancement of medicine.
  - 1 slide

History

- Mendel
  - Mendelian Genetics
- Watson & Crick
  - Structure of DNA
- Human Genome Project



In 1865, an Augustinian monk named Gregor Mendel proposed a theory of inheritance, before the discovery of the gene. Mendel did this through plant hybridization experiments. Mendel looked at seven different features of the plants: flower color (purple or white), seed and pod colors (yellow or green), where the flowers grew on the plant (axial or terminal), seed skin texture (smooth or wrinkled), pod formation (inflated or pinched), and stem height (long or short). By crossing the plants with different characteristics, Mendel found that patterns of inheritance of each of the characteristics could be predicted. These patterns came to be known as Mendelian genetics. Although Mendel was not recognized during his lifetime, he is now said to be the father of genetics.

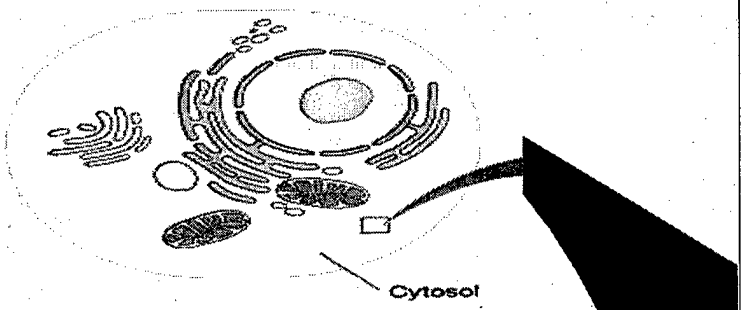
In 1953, James Watson and Francis Crick published their discovery of the structure of DNA (i;.e., the double helix). This was a landmark discovery as knowledge of the structure of DNA led to the elucidation of it's function.

The 3rd major revolution in genetics began in 1988 with the development of the Human Genome Project (HGP). This led a worldwide collaboration to sequence the entire human genome. This goal met ahead of schedule in April 2003, 50 years after the discovery of the structure of DNA by Watson and Crick.

Lesson 2 – Molecular Genetics

- This lesson encompasses the structure and function of DNA, RNA, and gene
  - 8 slides

Cellular organelles



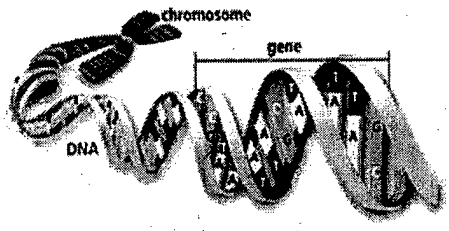
(b) Eukaryotic cell

© Addison Wesley Longman, Inc.

Yellow = ribosome – builds amino acid chains
Purple = nucleus – houses chromosomes
Red = mitochondria – produces energy

Genes and DNA

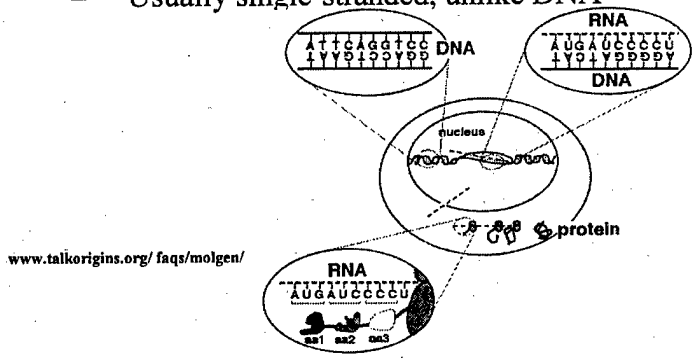
- Deoxyribonucleic acid (DNA)
  - Contains genetic instructions
  - Forms a double helix
- Genes
  - Long stretches of DNA
  - Arranged along chromosomes



DNA (deoxyribonucleic acid) is composed of repeating units that contains, within its structure, the instructions that tell our bodies how to grow, develop, and function. DNA enables the transmission of genetic information from parents to offspring in the form of genes. Genes are long stretches of DNA arranged along tiny, threadlike structures called chromosomes. Each DNA molecule is composed of two individual stands twisted into a ladder-like structure called a double helix. The DNA double helix is wound around special types of proteins and then further compacted to form chromatin, which continues to coil to create the highly condensed chromosome.

DNA→RNA →Protein

- DNA = blueprint for making proteins (proteins are a chain of amino acids – sequence of amino acid determines function/structure of protein)
- Ribonucleic acid (RNA)
  - Molecular link between DNA and proteins
  - Usually single-stranded, unlike DNA

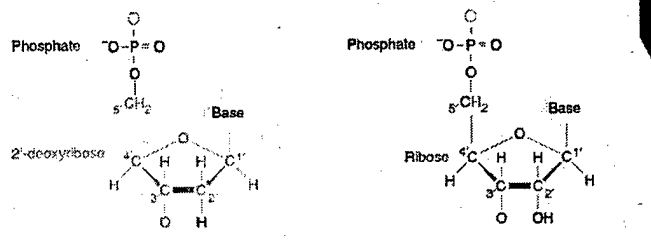


www.talkorigins.org/ faqs/molgen/

The information encoded in DNA is used to make proteins. You can think of DNA as blueprint used to make a temporary structure, that structure being ribonucleic acid (RNA). RNA is very similar to DNA, and it plays an instrumental role in the process of protein synthesis. RNA is the molecular link between the genetic code of DNA and the amino acid sequence of proteins. The flow of information from DNA to RNA to protein occurs in the following manner. DNA directs the synthesis and sequence of RNA while RNA directs the synthesis and sequence of protein. Whereas DNA exists as a double helix, RNA is usually a single-stranded molecule.

Components of DNA and RNA

- DNA and RNA are polymers made of nucleotides, which contain 3 chemical entities
  - Phosphate group
  - Sugar molecule (deoxyribose in DNA and ri[illegible]e in RNA)
  - Nitrogen-containing base (A, T, C and G in DN[illegible] U, C, and G in RNA)



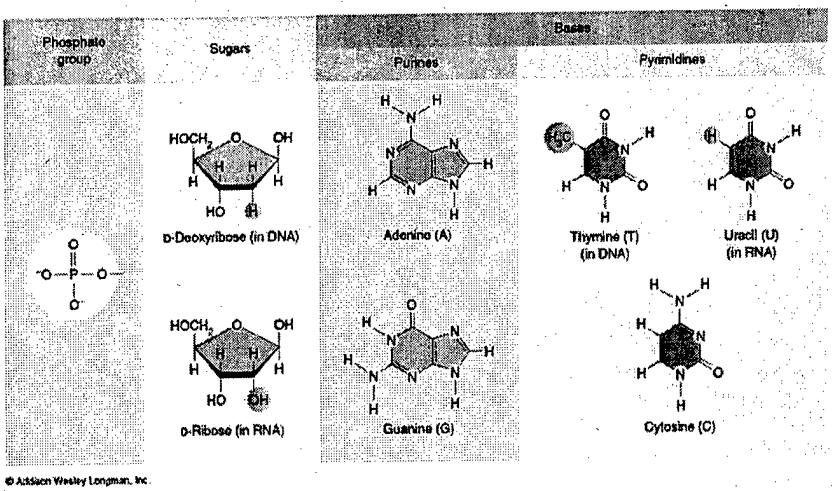
(a) Repeating unit of deoxyribonucleic acid (DNA)

© Addison Wesley Longman, Inc.

(b) Repeating unit of ribonucleic acid (RNA)

Both DNA and RNA are chains made up of many individual nucleotides. The three building blocks of a nucleotide are: a phosphate group, a five-carbon sugar, and a nitrogen-containing base. DNA contains deoxyribose as its sugar, and the bases adenine (A), thymine (T), C (cytosine), and G (guanine). In contrast, RNA contains ribose as its sugar, and the bases adenine (A), uracil (U), cytosine (C), and guanine (G). It is the linear order of the bases in a DNA molecule that provide the genetic code for building a protein.

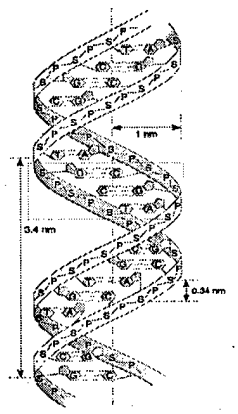
Purines and Pyrimidines

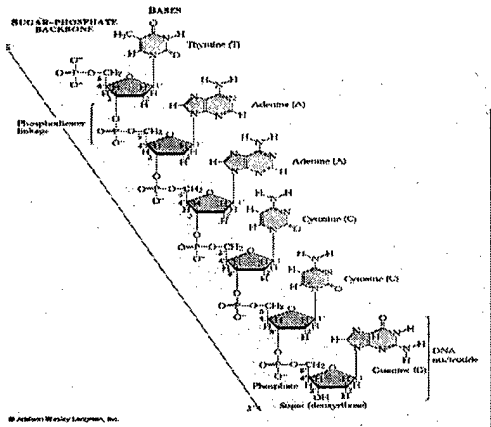


The nitrogen-containing bases in DNA and RNA are categorized as either purines (adenine and guanine) or pyrimidines (thymine, uracil, and cytosine) based on their chemical structure.

DNA Structure

- Key features of DNA
  - Double helix
  - Bases joined by hydrogen bonds (weak)
  - Phosphodiester backbone (strong)

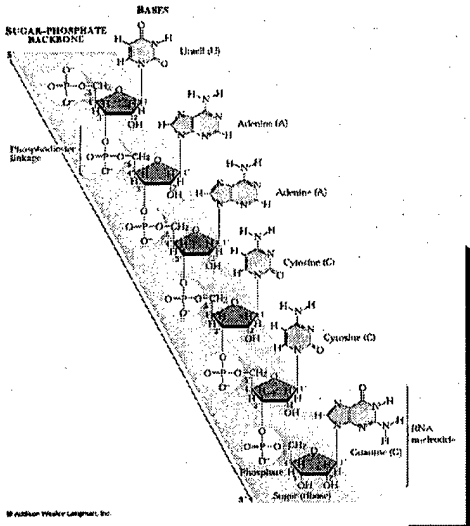




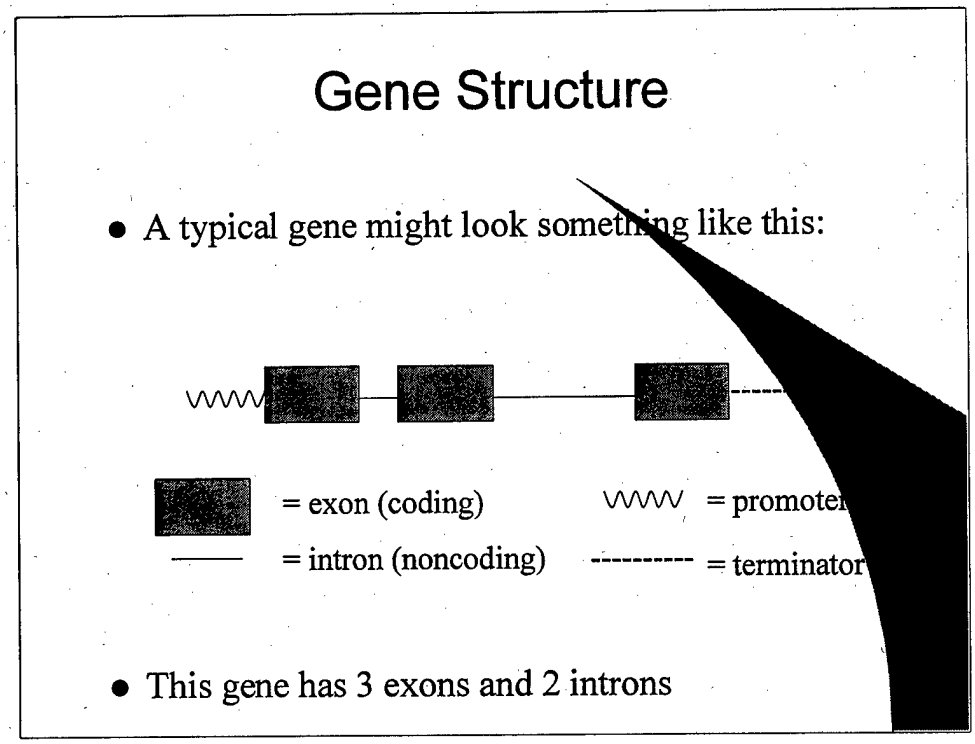
A strand of DNA will come together (hybridize) with a complementary strand of DNA to form double-stranded DNA. In this double helix structure, the bases in each nucleotide pair up and are link together by weak hydrogen bonds. Specifically, A bonds with T and C bonds with G. Two hydrogen bonds exist between A and T (A=T) while three exist between G and C (C ≡ G). Therefore, DNA that is "GC" rich will have a stronger bond and be harder to separate. Each strand of DNA has direction, 5' to 3' (read 5 prime to 3 prime). The complementary strands run opposite of one another so that one is 5' to 3' while the other is 3' to 5'. The DNA resembles a twisted ladder, in which the base pairs form the rungs and the sugar phosphate groups form the sides. Adjacent deoxyribose units are joined by phosphodiester bonds, which are very strong.

RNA Structure

- Key features of RNA
  - Sugar→ribose
  - Uracil, not thymine
  - Usually single-stranded
  - Phosphodiester backbone



The overall chemical structure of RNA is very similar to DNA, except that the sugar ribose is ribose instead of deoxyribose and uracil is substituted for thymine. Unlike DNA, RNA should remain single stranded. However, sometimes RNA will find a complementary strand of RNA to bind to or fold back on itself to create double-stranded RNA. Double-stranded RNA structures can form within a single RNA molecule or between two separate RNA molecules.



A gene is a section of DNA on a chromosome that codes for a protein. A typical gene has several key components. Exons are the regions of a gene that contain the bases that are utilized in coding for a protein. Introns are the regions of a gene that contain the bases that are not used in coding. Intronic sequences intervene between the exons and are not part of the final protein because they are spliced out. Bases in the promoter provide a signal to tell the cell's machinery where to begin transcription (the process of synthesizing RNA from the DNA template). The promoter is usually located before or within a gene. for proteins and intervene between the exons. The teminator bases of a gene supply a signal that helps the cell's machinery determine where to stop transcription. The terminator is typically found at the end of a gene.

Summary Questions

Which of the following is true when comparing DNA and RNA?

A) DNA is usually double stranded, whi RNA is usually single stranded
B) RNA does not contain thymine, but DNA
C) RNA contains uracil, but DNA does not
D) All of the above are true when comparing DN and RNA

Is the following sequence DNA or RNA?

5'-GGACCGUAAGGC-3'

A) DNA
B) RNA

Answers

Which of the following is true when comparing DNA and RNA?

A) DNA is usually double stranded, whi RNA is usually single stranded
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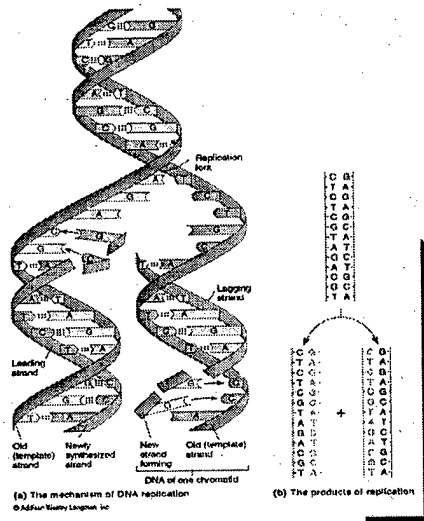
A) DNA
B) RNA

Lesson 3 – DNA Replication

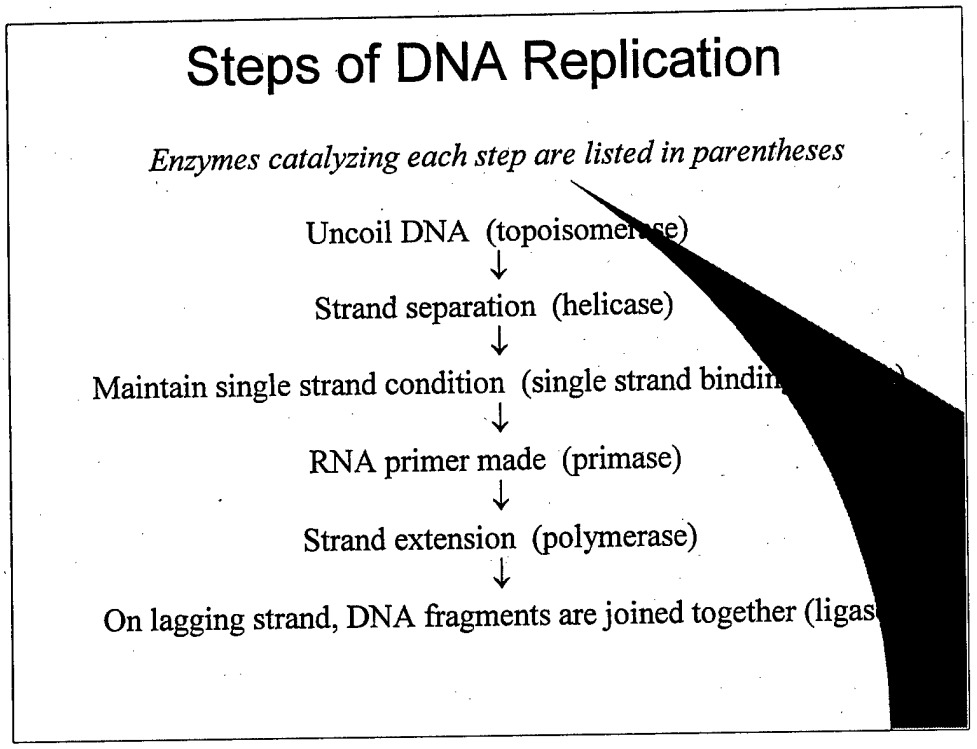
- This lesson covers the process of DNA replication
  - 5 slides

DNA Replication

- Results in two identical daughter molecules, each containing
  - 1 parental strand (brown)
  - 1 newly synthesized strand (blue)
- Referred to as semiconservative replication

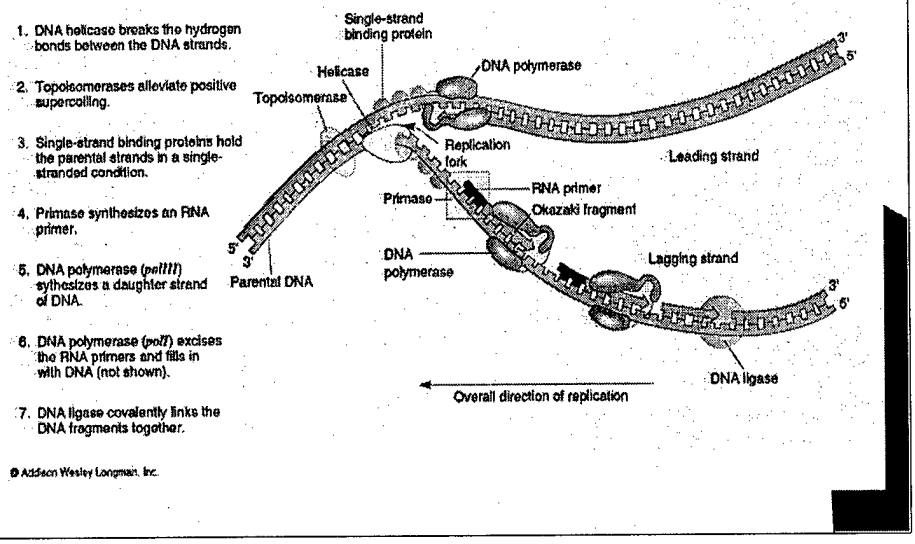


DNA replication produces two copies of DNA with the same sequence as the original DNA molecule. The original double-stranded DNA opens up and each parental strand acts as a template to make a new strand – 2 double-stranded pieces from one double-stranded. The new strand is easy to build because you always know the base sequence of the other strand – remember A always pairs with T and C always pairs with G. This type of replication is called semiconservative.

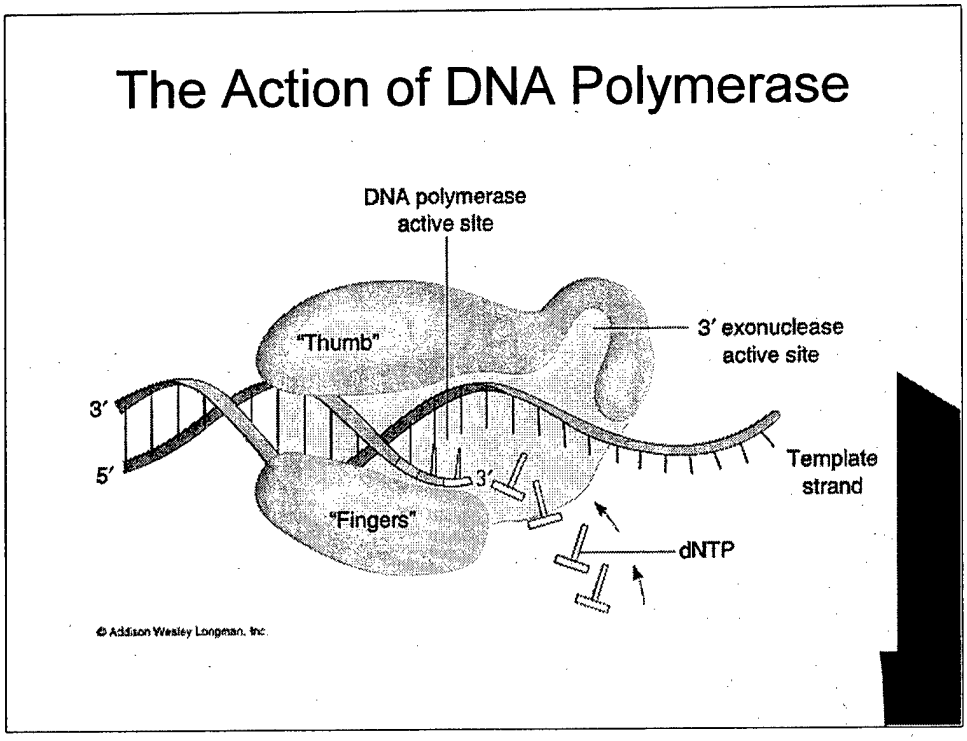


The process of DNA replication involves multiple steps each catalyzed by a specific enzyme.

Pictorial Representation of DNA Replication

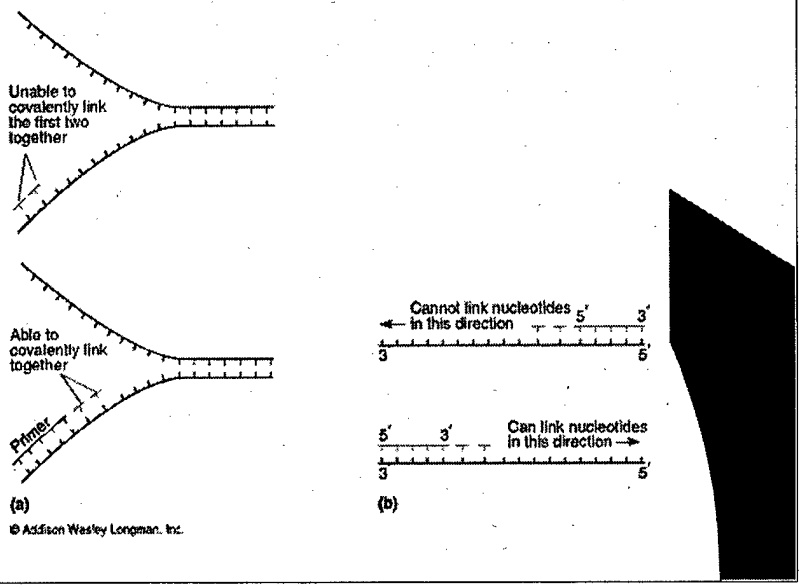


During the process of DNA replication, note that one strand is made continuously and is called the leading strand, while the other strand needs to be made in pieces (called Okazaki fragments) and is referred to the lagging strand. One strand needs to be made in pieces because the polymerase that adds nucleotides can only work by making the new strand in the 5' to 3' direction (only works in one direction).



DNA polymerase slides along the template strand, and it synthesizes new strands by connecting nucleotides in a 5' to 3' direction. The structure of DNA polymerase resembles a hand that is wrapped around the template strand. In this regard, the movement of DNA polymerase along the template strand is similar to a hand that is sliding along a rope. DNA polymerase catalyzes the formation of a covalent bond between the 3'-OH group on the previous nucleotide and the innermost 5'-phosphate group on the incoming nucleotide.

Unusual Features of DNA Polymerase



There are several unusual features of DNA polymerase function. First, DNA polymerase can only elongate a strand starting with a short RNA primer. Second, DNA polymerase can only attach nucleotides in a 5' to 3' direction. Note the template strand is in the opposite, 3' to 5', direction.

Summary Questions

What is a correct order of events you would expect during DNA replication?

A) Strands separate, RNA primer binds, strand extension, fragments ligated
B) RNA primer binds, DNA uncoils, strands separate, strand extension
C) RNA primer binds, strands separate, DNA uncoils, strand extension
D) Strands separate, strand extension, RNA primer binds, fragments ligated

What would the complementary strand of this DNA look like:

5'- GGCAGCTTGCCA -3'

A) 3'- CCGTCGAACGGT -5'
B) 5'-CCGTCGAACGGT -3'
C) 3'-GGCAGCTTGCCA -5'
D) 3'- CCGUCGUUGCCU -5'

Answers

What is a correct order of events you would expect during DNA replication?

A) Strands separate, RNA primer binds, strand extension, fragments ligated
B) RNA primer binds, DNA uncoils, strands separate, strand extension
C) RNA primer binds, strands separate, DNA uncoils, strand extension
D) Strands separate, strand extension, RNA primer binds, fragments ligated

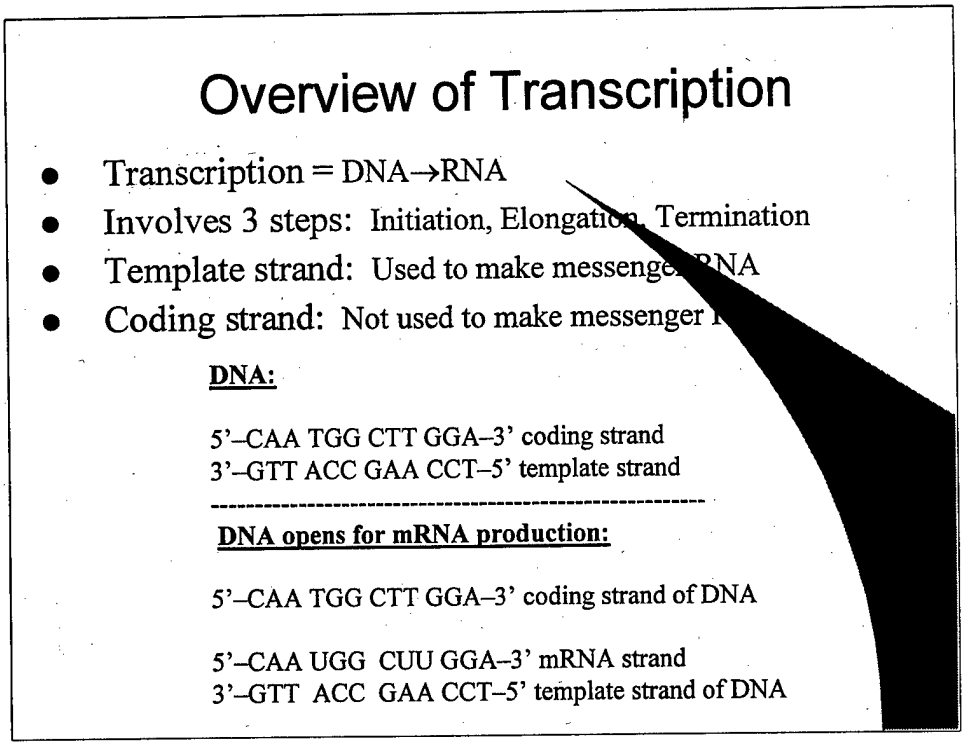
What would the complementary strand of this DNA look like:

5'- GGCAGCTTGCCA -3'

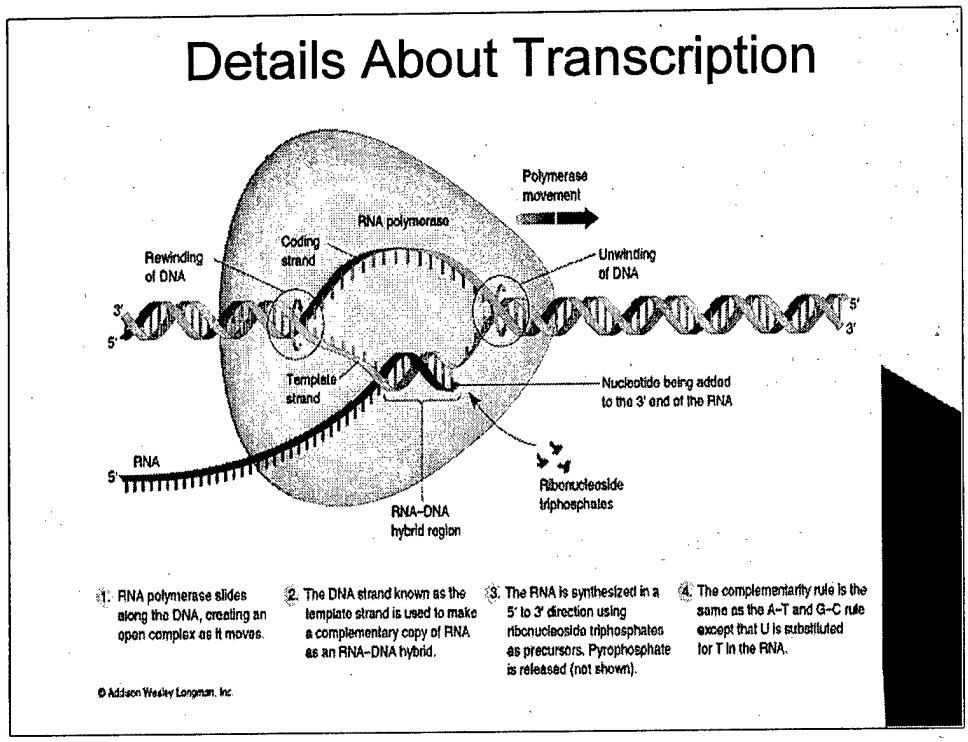
A) 3'- CCGTCGAACGGT -5'
B) 5'-CCGTCGAACGGT -3'
C) 3'-GGCAGCTTGCCA -5'
D) 3'- CCGUCGUUGCCU -5'

Lesson 4 – Protein Synthesis

- This lesson includes information regarding the processes of transcription, RNA modification, and translation
  - 10 slides

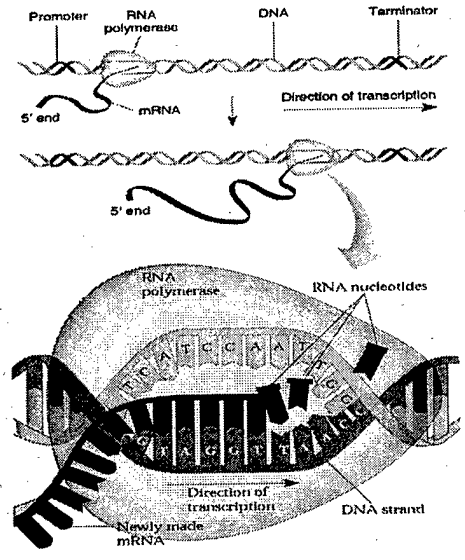


Transcription occurs when the molecular machinery in the cell opens the double-stranded DNA and reads the genetic code to make messenger RNA (mRNA). There are three stages of transcription: initiation, elongation, and termination. The strand of DNA that is accessed to make mRNA is called to template strand. The other strand of DNA is considered the coding strand because the new RNA strand will be identical to this strand (with the exception of having U's for T's).

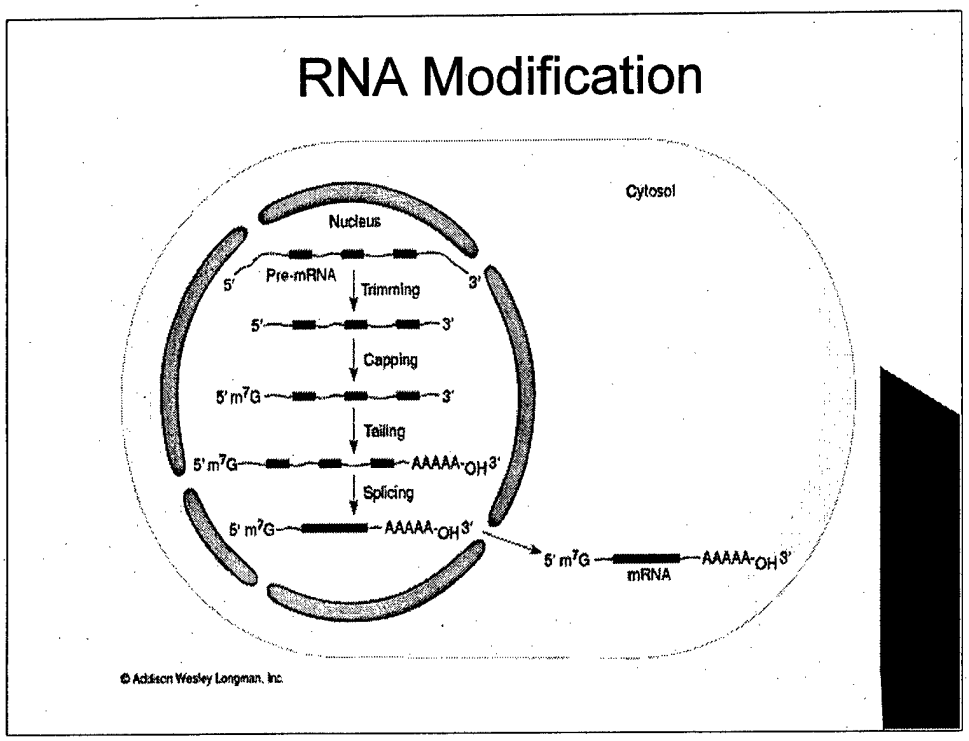


This slide illustrates the initiation and elongation phases of transcription in more detail. The initiation phase of transcription begins when specific proteins called transcription factors bind to the promoter of a gene. Then, the RNA polymerase enzyme recognizes the promoter and slides along the DNA template to unwind and open the double helix. During the elongation (synthesis) phase, RNA polymerase synthesizes a complementary copy of RNA using single-stranded DNA as a template and ribonucleoside triphosphates as precursors.

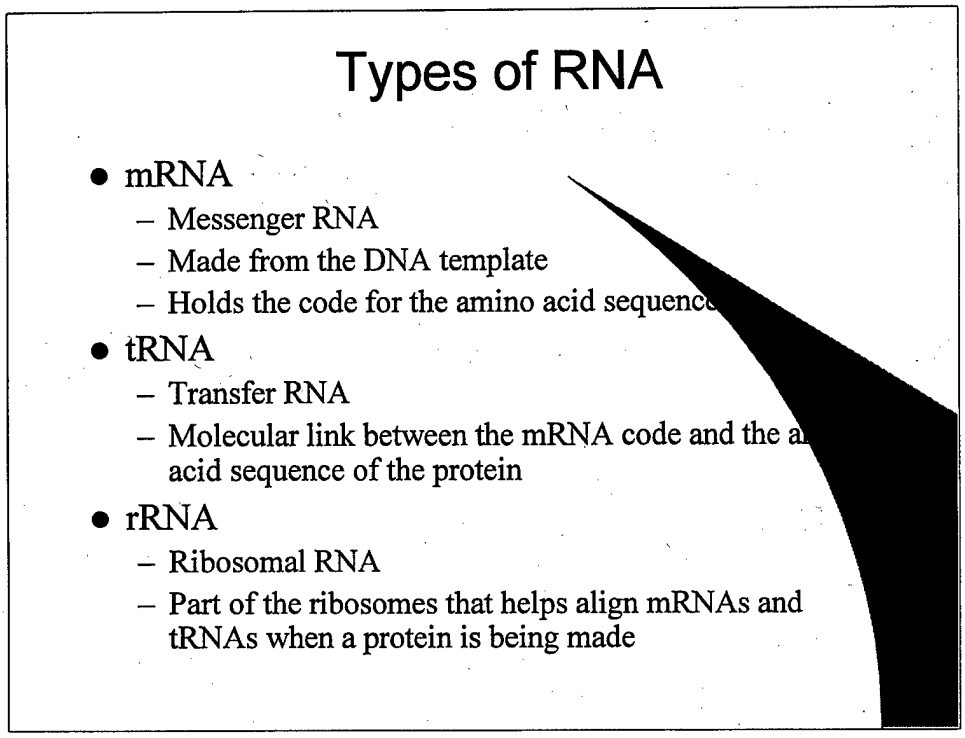
More Details About Transcription



The new strand is new strand of RNA is made in a 5' to 3 direction. The termination phase occurs when RNA polymerase encounters termination signals and, consequently, drops off the DNA template. The end product of transcription is the creation of a strand of mRNA. This mRNA is, in turn, used as a template to make protein through a separate process called translation.



After an mRNA molecule is made but before translation occurs, the immature mRNA strand undergoes several chemical modifications. These modifications all take place in the nucleus of a cell and are necessary so that the mRNA can localize to the ribosome in the cytoplasm for translation. They also influence mRNA stability and allow for the removal of introns. Trimming involves the removal of excess bases from the 5' and 3' ends of the mRNA. Capping refers to the addition of a methylated G to the 5' end of the mRNA and enables the RNA to localize to the ribosome. Tailing occurs when a string of A's is added to the 3' end of the mRNA (called the poly A tail). The more A's there are, the more stable the mRNA. During the splicing, the introns are removed from the mRNA, and the exons are reconnected prior to the mRNA exiting the nucleus. Collectively, these modifications create a mature mRNA molecule that is ready to participate in the translation process.

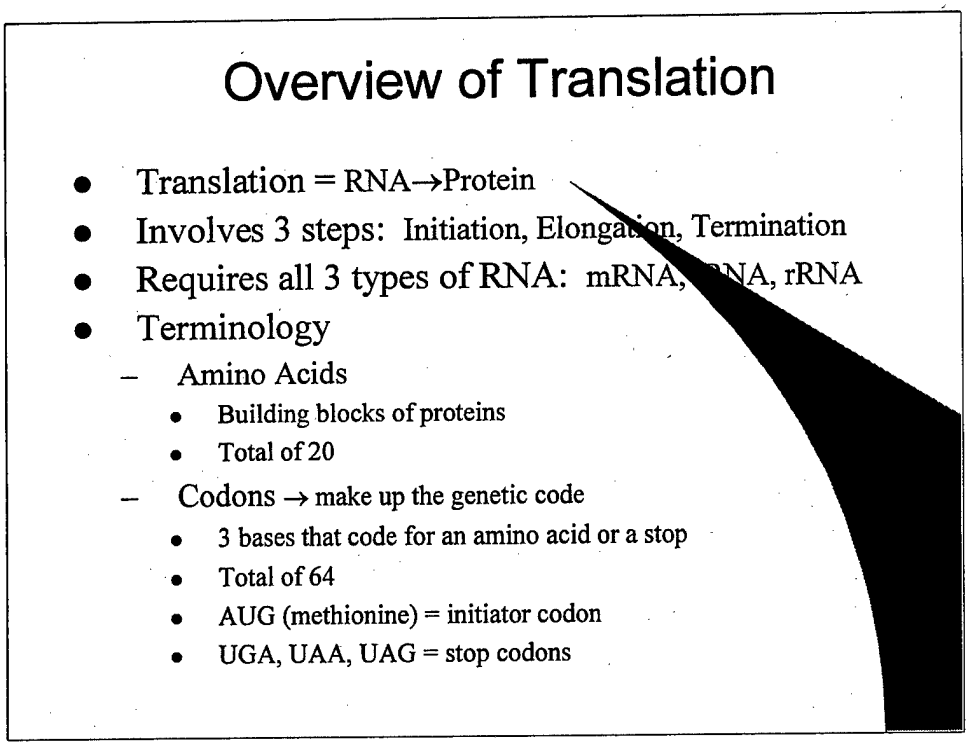


In addition to mRNA, two other types of RNA are needed for protein synthesis: tRNA and rRNA.

As discussed previously, messenger RNA (mRNA) is synthesized from DNA and carries coded information. After the necessary chemical modifications are made, mRNA is transported from the nucleus to the cytoplasm, where it is decoded (translated) to determine the amino acid sequence of the corresponding protein.

Transfer RNA (tRNA) is the molecule that recognizes the mRNA code, docks at the appropriate position on the mRNA, and bring with it (or transfers) the appropriate amino acids for building a protein. Each tRNA molecule is specific for a particular amino acid.

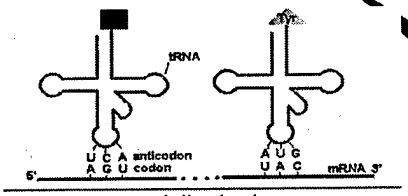
Ribosomal RNA (rRNA) constitutes part of the ribosomes and is involved with translating the mRNA into a protein by aligning the appropriate mRNAs and tRNAs.



Translation is the process of using mRNA as a template to make the peptide chain that will eventually become the mature protein. Just like in transcription, there are three stages of translation: initiation, elongation, and termination. It is important to point out that translation requires all three types of RNA (mRNA, tRNA, and rRNA).

The key to translation is the genetic code that relates specific amino acids to combinations of three adjacent bases along the mRNA. The complete genetic code is shown in the next slide. But, to summarize, each three base unit of mRNA is called a codon. Each codon either encodes a particular amino acid or signals the end of translation. Amino acids are the building blocks of proteins, and there are 20 in all. However, there are actually 64 possible codons that make up the genetic code. Thus, most amino acids are specified by more than one codon and the code is said to be degenerate. In this code AUG (coding for the amino acid methionine) is always the initiator codon, and UGA, UAA, and UAG are the stop codons.

The Genetic Code

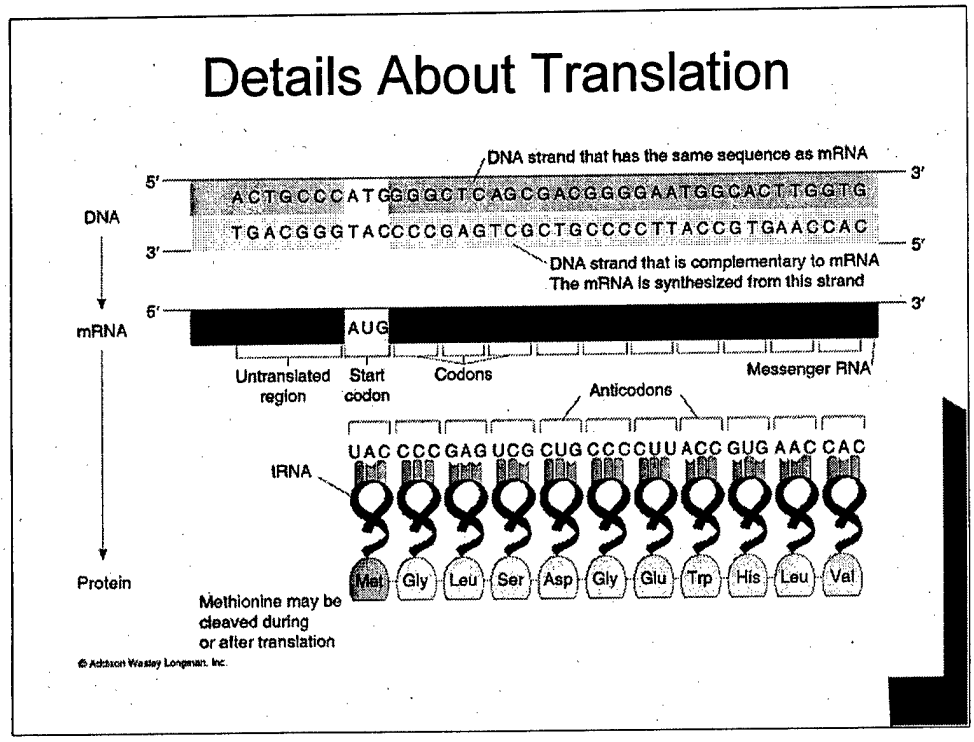


2nd base in codon

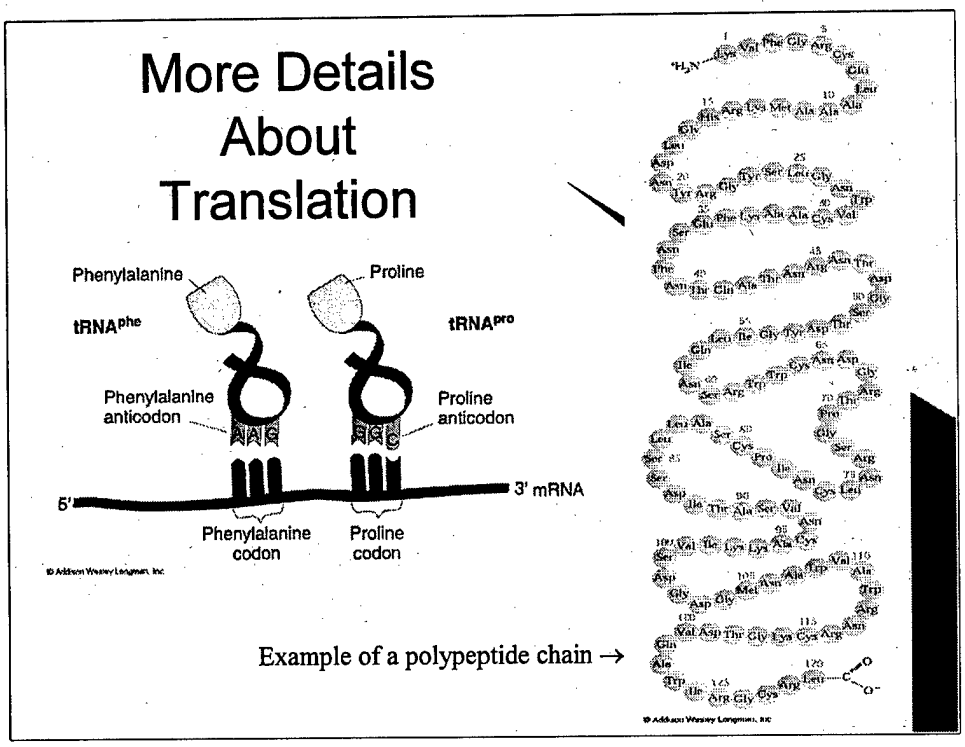
1st base in codon	U	C	A	G	3rd base in codon
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

The Genetic Code

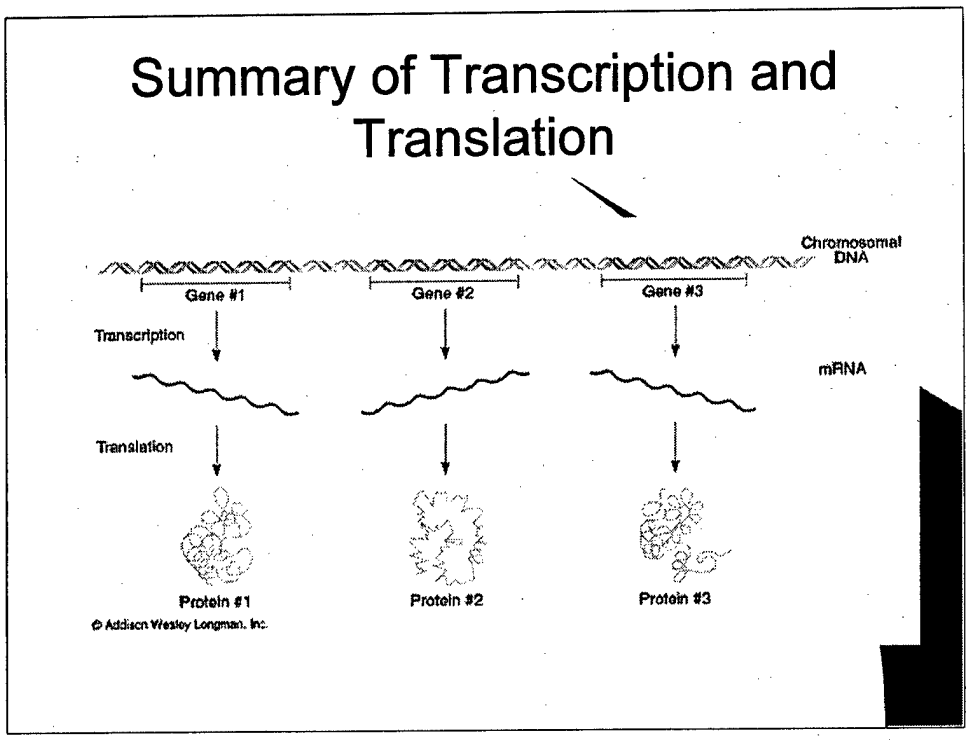
This is the complete genetic code.



Protein synthesis occurs in the cytoplasm on specific organelles called ribosomes that have binding sites for all the interacting molecules. Initiation begins when mRNA and tRNA meet at the ribosome and the start codon (AUG) is recognized. Elongation (synthesis) involves the building of the amino acid chain. Adjacent amino acids are connected by peptide bonds. Termination occurs when a stop codon is reached in the mRNA and the amino acid chain are released from the ribosome. The end product of translation is an amino acid chain, which is also called a primary protein.



The process of translation is dependent upon precise recognition between tRNAs and mRNA. A specific site on each tRNA molecule forms a three base anticodon that binds to a complementary sequence (i.e., the codon) in the mRNA. At its other end, the tRNA carries the amino acid that corresponds to the codon in the mRNA according to the genetic code. This amino acid is then added to the growing polypeptide chain. As shown, a protein (polypeptide) consists of many individual amino acids.



This diagram summarizes the process of transcription and translation. Each gene encodes a protein that has its own distinctive structure and function.

First, the sequence of nucleotides within the DNA is transcribed to make a complementary sequence of nucleotides, known as mRNA. Second, the sequence of nucleotides in mRNA is translated into a sequence of amino acids within a polypeptide. tRNA molecules acts as intermediates in this translation process.

Summary Questions

Transcribe this template strand of DNA into its complimentary mRNA : 3'-GCCTATAAA[illegible]AG-5'

A) 5'-CGGATATTTGTC-3'
B) 5'-CGGUTUTTTGTC-3'
C) 5'-CGGAUAUUUGUC-3'
D) 5'-CGGUAUAAAGAC-3'

Using DNA as a template and producing mRNA describ[illegible] process called

A) translation
B) replication
C) peptide synthesis
D) transcription

Answers

Transcribe this template strand of DNA into its complimentary mRNA : 3'-GCCTATAAA[illegible]AG-5'

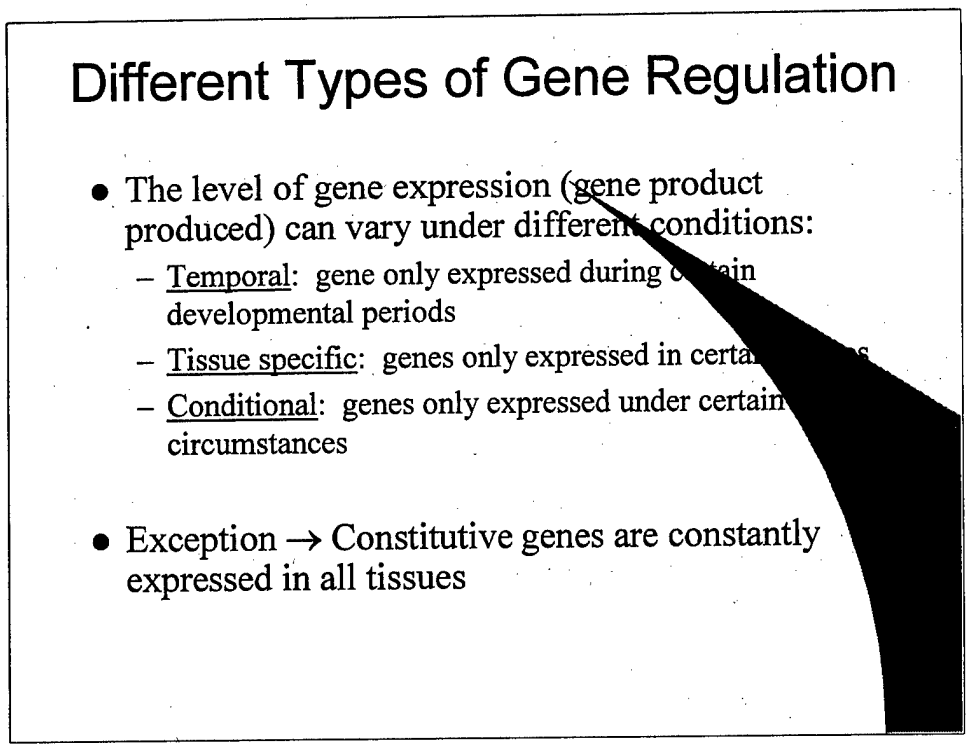
A) 5'-CGGATATTTGTC-3'
B) 5'-CGGUTUTTTGTC-3'
C) 5'-CGGAUAUUUGUC-3'
D) 5'-CGGUAUAAAGAC-3'

Using DNA as a template and producing mRNA describ[illegible] process called

A) translation
B) replication
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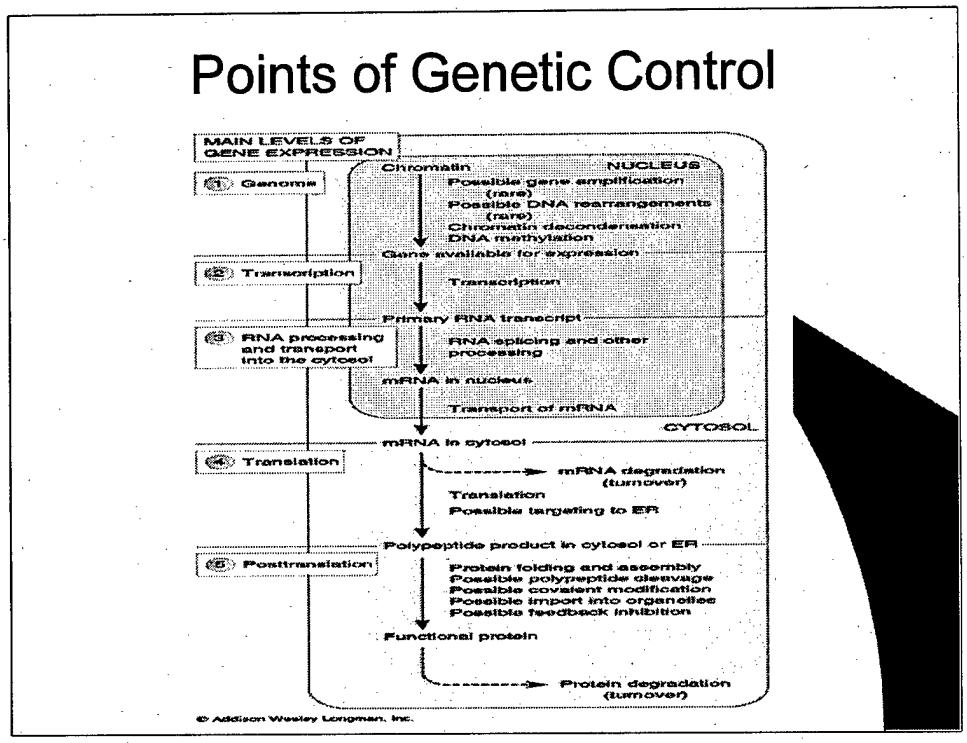
Lesson 5 – Gene Regulation

- This lesson outlines the complex processes which play a role in gene regulation
- Gene regulation refers to varying levels of products produced from different genes under varying conditions
  - 9 slides



As mentioned previously, genes encode proteins with diverse functions. There are approximately 30,000 genes in the human genome. The level of expression of most genes varies under different conditions due to a intricate set of interrelationships.

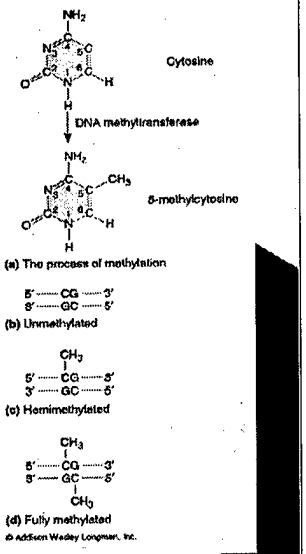
Gene regulation can be temporal, tissue-specific, or conditional in nature. Temporal regulation means that some genes are only expressed during certain developmental periods or particular times (e.g., after eating food). Tissue-specific regulation indicates that some genes are only expressed in certain tissues. Conditional regulation refers to the fact that some genes are only expressed under certain circumstances (e.g., if you are exposed to a virus). Although the expression of most genes is tightly controlled by a variety of mechanisms, an exception is constitutive genes. These are gene whose products are made constantly in all tissues at all times.



As this diagram depicts, the overall process of gene regulation in exceedingly complex. Gene regulation can occur at multiple points, including the DNA or chromatin level, the transcriptional level, the mRNA and translational level, and the post-translational level. In this lesson we will only touch upon the key concepts.

Regulation at the DNA or Chromatin Level

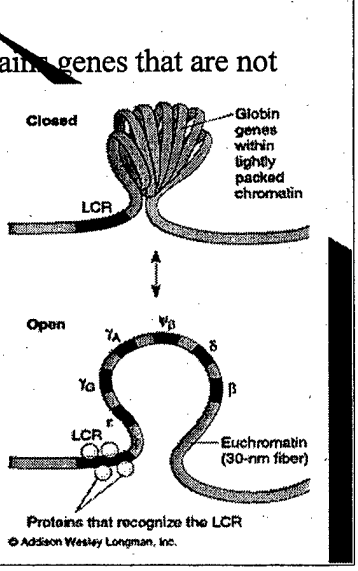
- Methylation
  - Attachment of a methyl group to certain C's in CG pairs of the DNA
  - This chemical modification inhibits transcription, and results in the gene not being expressed
- Housekeeping genes
  - Rarely methylated and therefore the gene product made ubiquitously
- Tissue-specific genes
  - Usually methylated to some extent in cells where the gene product is not needed



DNA methylation is a chemical process that modifies certain cytosine nucleotides and, in this way, inhibits transcription. An enzyme attaches a methyl group to the number 5 carbon on cytosine in certain CG dinucleotide pairs. A CG sequence in DNA can be unmethylated (methylated on neither strand), hemimethylated (methylated on one strand), or fully methylated (methylated on both strands). Housekeeping genes code for proteins required by most cells and are rarely methylated. Tissue specific genes code for proteins only utilized by certain cells. These types of genes are highly regulated and usually methylated to some degree in cells where the gene product is not needed.

Regulation at the DNA or Chromatin Level

- Chromatin conformation
  - Tightly compacted chromatin contains genes that are not actively transcribed
  - Loosely compacted chromatin contains genes that are more actively transcribed
- Gene amplification
  - Copies of a gene increase
- Minichromosome
  - Many copies of a gene arranged into a circular DNA molecule



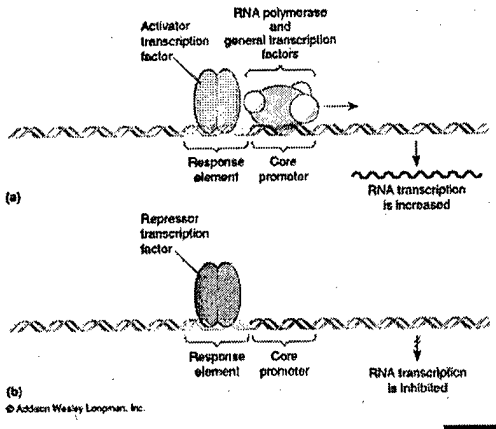
There are also several other types of gene regulation that have an effect at the DNA or chromatin level. Chromatin refers to the complex of DNA and proteins of which chromosomes are composed. How DNA and genes are packaged into chromosomes influences gene expression. Chromatin that is tightly compacted contains genes that are not actively transcribed, whereas loosed compact chromatin contains genes that are more actively transcribed.

Gene amplification occurs when there are many additional copies of a gene or section of genome present in a cell. It is a common occurrence in many cancers, such as colorectal cancer and squamous cell carcinoma of the head and neck.

A minichromosome is a circular molecule of DNA containing many copies if a particular gene. Both gene amplification and the formation of minichromosomes are abnormal and relatively rare events; however, they can disrupt gene regulation.

Regulation at the Transcriptional Level

- Repressor
  - Protein that inhibits transcription
- Activator
  - Protein that increases transcription
- Inducer
  - Small molecule that indirectly increases transcription

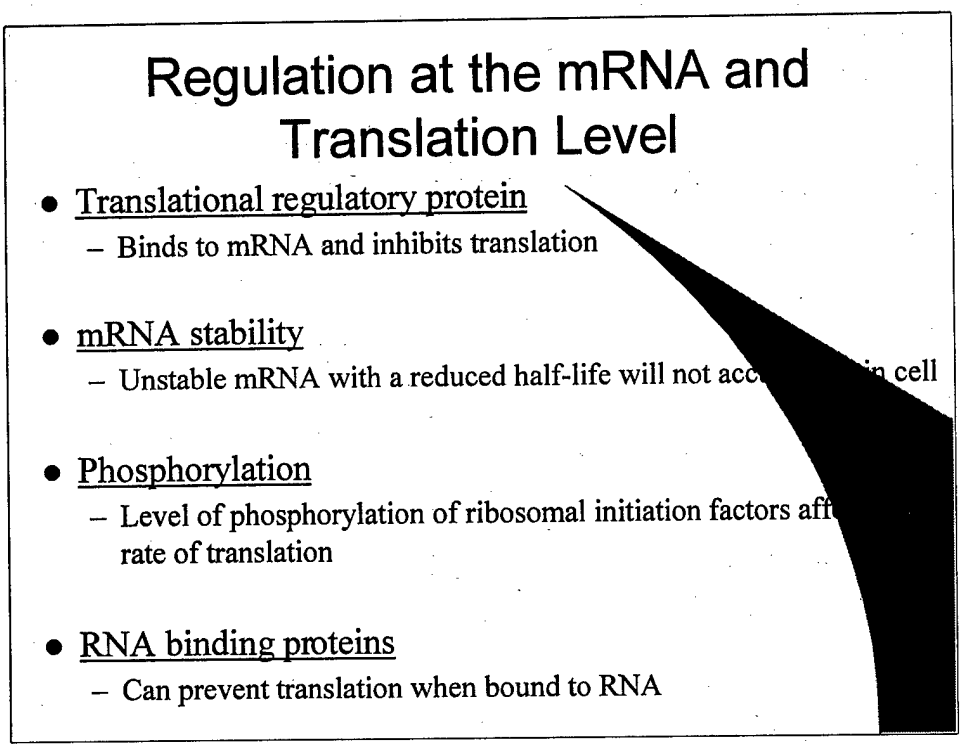


➔Genes regulated by inducer molecules are called *inducible ge*

Several elements influence gene expression by altering the rate of transcription. A repressor is a protein that binds to DNA and inhibits transcription. It therefore represents a type of negative control.

On the other hand, an activator is a protein that bind to DNA and increases transcription. Thus, it represents a type of positive control.

An inducer is a small molecule that indirectly causes transcription to increase in one of two ways. First, it may bind to an activator and enhance its binding affinity for DNA. Second, it may form a complex with a repressor protein and prevent it from binding to DNA. Genes that are regulated by inducer molecules are called inducible genes.



Sometimes the rate at which mRNA is made is influenced by certain factors that consequently impact the rate of protein production. There are a number of examples of gene regulation at this level. Translational regulatory proteins can bind to the mRNA sequence and inhibit translation by, for instance, blocking the ribosome's ability to get to the start codon.

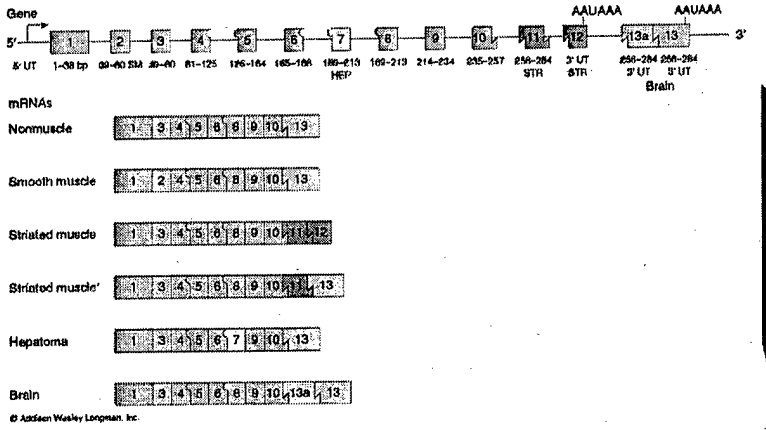
The stability of the mRNA molecule also affects the efficiency of translation. An unstable mRNA has a reduced half-life and does not accumulate in a cell.

In addition, the rate of translation is influenced by the level of phosphorylation of ribosomal initiation factors.

Also, RNA binding proteins have the ability to prevent translation when they are bound. because an unstable mRNA

Regulation at the mRNA and Translation Level

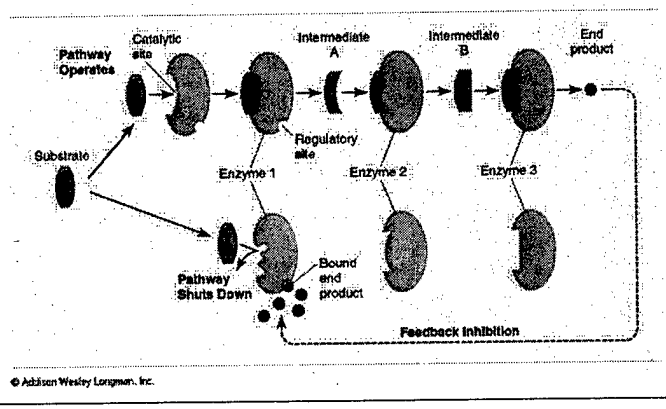
- Alternative splicing
  - Certain splice sites in a gene are preferred in different tissues



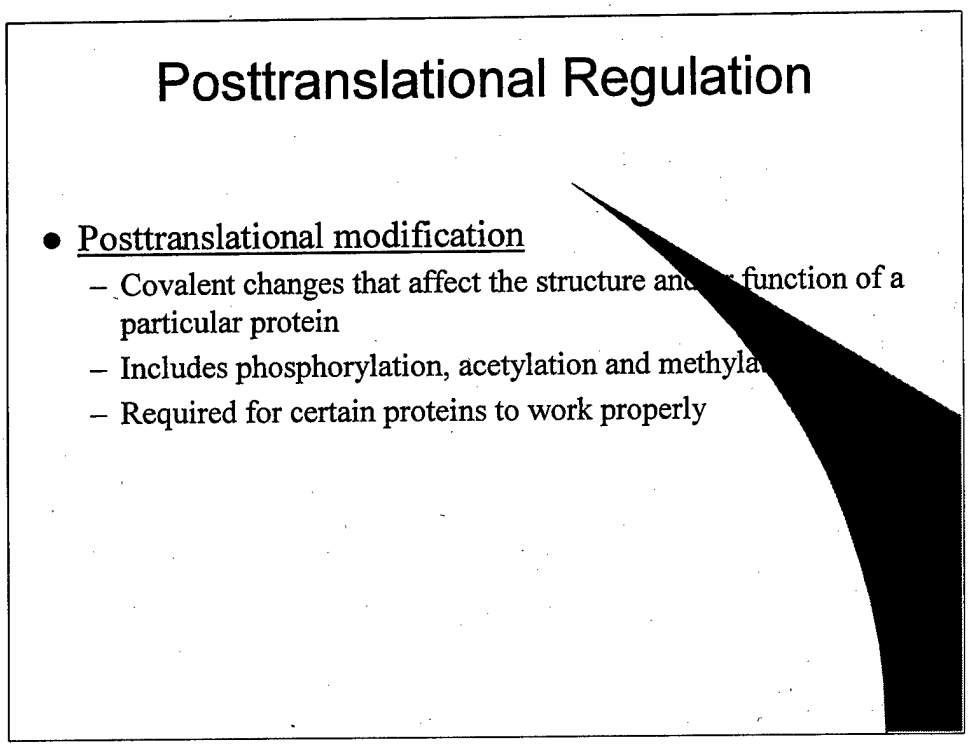
Splice sites are the positions at which the introns are cut and removed from the pre-mRNA and the exons are joined together. For some genes, it has been observed that certain splice sites within the gene are preferred in different tissues. The use of alternative splice sites to form the mature mRNA can result in differences in gene expression, as well as difference in the structure of the protein product. As shown here, alpha-tropomyosin mRNA can be spliced multiple ways depending on the type of tissue in which it is found. The top part of this figure depicts the gene structure of the alpha-tropomyosin gene. Exons are shown as colored boxes, and introns are illustrated as connecting black lines. The lower part of the figure describes the final mRNA products in various types of cells.

Posttranslational Regulation

- Feedback inhibition
  - Protein produced by a pathway inhibits an enzyme that acts early in the pathway
  - Reduces further protein production



Gene expression can also be regulated at the posttranslational level. One example of this type of gene regulation is feedback inhibition. In this process, a protein that is synthesized by a particular pathway inhibits an enzyme that catalyzes a step early in the pathway. The ultimate result is a reduction in further protein production. The figure illustrates feedback inhibition in a metabolic pathway. The substrate is converted to a product by the sequential action of three different enzymes. Enzyme 1 has a catalytic site that recognizes the substrate, and it also has a regulatory site that recognizes the product. When the product binds to the regulatory site, it inhibits enzyme.



A second example of regulation that occurs after the formation of a protein is called posttranslational modification. This refers to covalent changes that affect the structure and/or function of a resultant protein. The major types of posttranslational modification are phosphorylation, acetylation, and methylation. Even though protein is made, it may not be functional without further modifications, such as these.

Summary Questions

Methylation of DNA

A) is not found in humans
B) induces transcription
C) inhibits transcription
D) up regulates translation

True or False: Alternative splicing does not occur in humans

A) True
B) False

Answers

Methylation of DNA

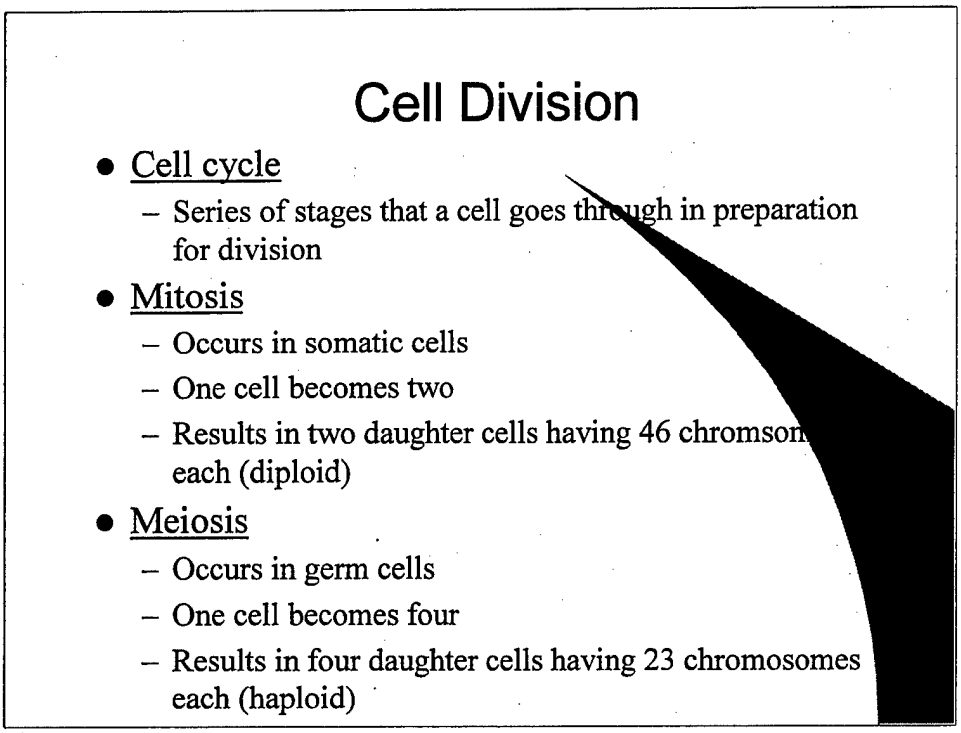
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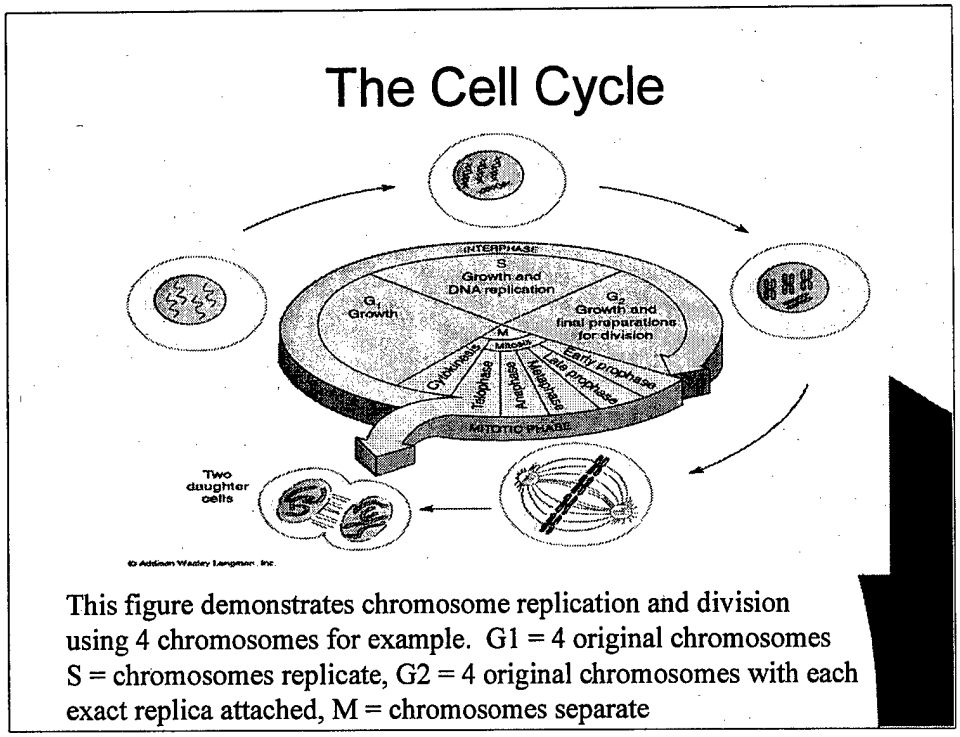
Lesson 6 – The Cell Cycle

- This lesson covers the basics of the cell life cycle, which includes mitosis in somatic cells (all cells of the body with the exception of germ cells) and meiosis in germ cells (cells that produce gametes)
  - 10 slides



Cells replicate by dividing. The cell cycle is a series of stages a somatic cell goes through in preparation for division. Division occurs by either mitosis or meiosis. When a zygote is formed, it must divide to become the embryo and hence the developing fetus. This is achieved through a process called mitosis. The cells that undergo mitosis are referred to as somatic cells, meaning they are the cells that make the organism function. In mitosis, one cell becomes 2, 2 become 4, 4 become 8, 8 become 16, and so forth. Each new "daughter" cell contains an identical set of 46 chromosomes, which are the same as that of the original cell. These cells are called diploid, indicating that they have a total of 46 chromosomes.

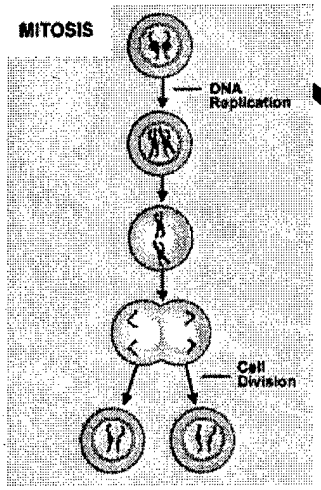
Meiosis refers to a different process that results in the formation of the egg and sperm. In this process, the number of chromosomes is eventually divided in half, so that the daughter cells contain only 23 chromosomes instead of 46 chromosomes. These cells are called haploid, meaning that they have which a total of 23 chromosomes, which is half of the diploid number.



This figure demonstrates chromosome replication and division using 4 chromosomes for example. G1 = 4 original chromosomes S = chromosomes replicate, G2 = 4 original chromosomes with each exact replica attached, M = chromosomes separate

Somatic cells that are destined to divide progress through a series of stages, denoted G1, S, G2, and M phases (mitosis). This diagram shows the progression of a cell through mitosis to produce two daughter cells. The original diploid cell had two pairs of chromosomes, for a total of four individual chromosomes. During Gap 1 (G1) phase the cell carries out the functions for which it was created. During the DNA synthesis (S) phase, all the DNA in a cell is replicated so the cell then contains two copies of each chromosome (sister chromatids) and, therefore, twice as much genetic material. During Gap 2 (G2) phase the cell functions until reaching mitosis (M). G1, S, and G2 comprise approximately 90-95% of the cell cycle and together are called interphase.

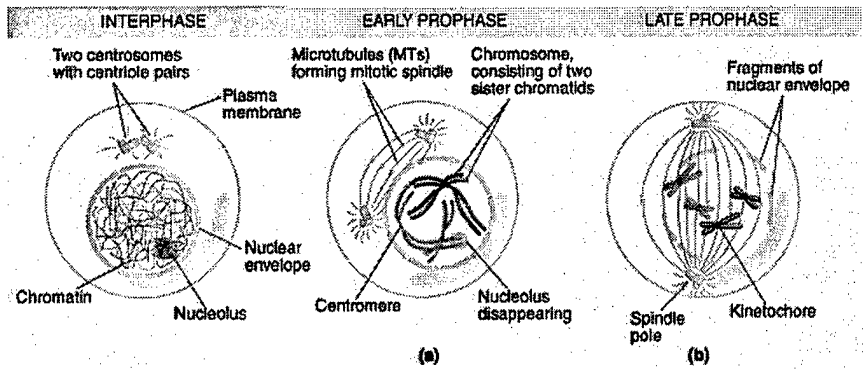
The Cell Cycle - Mitosis



http://genetics.gsk.com/graphics/mitosis-big.gif

The mitosis (M) phase of the cell cycle is when the chromosomes are distributed so that the 2 daughter cells that result from cell division get the correct genetic material. Specifically, the sister chromatids separate, resulting in two identical daughter cells each having 46 chromosomes. Mitosis occurs throughout the lifespan, results in 2 identical diploid cells from 1, and occurs in somatic cells.

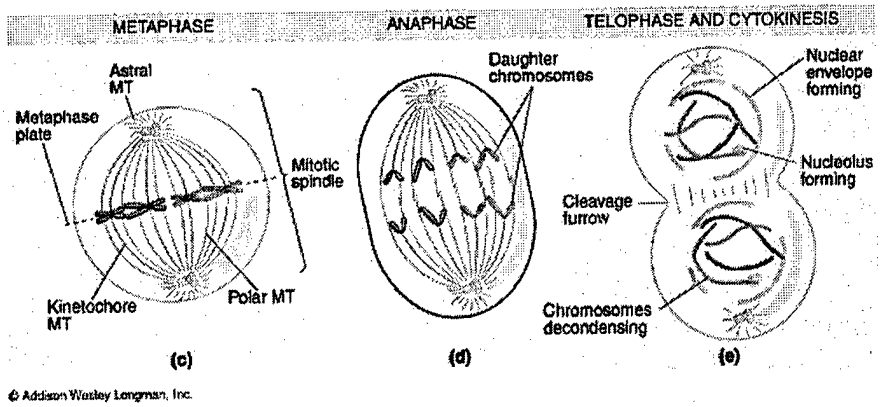
Mitosis - Interphase and Prophase



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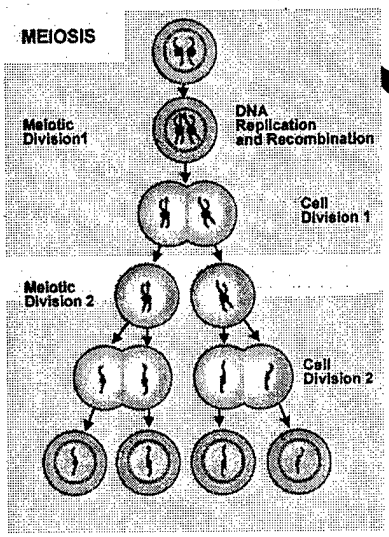
This figures shows the first part of the process of mitosis. The panels emphasize the sorting and separation of the chromosomes. In this case, the original diploid cell had four chromosomes (two in each set). At the start of mitosis, these have already replicated into eight sister chromatids.

Mitosis - Metaphase, Anaphase, Telophase, and Cytokinesis



These panels emphasize the sorting and separation of the chromosomes. Each chromosome travels to the center of the cell and aligns itself at the metaphase plate. Then, the spindle fibers that are attached to each chromosome contract, splitting the sister chromatids at the centromere. At the end of mitosis, the chromosomes in each of the 2 daughter cells contract and the nuclear envelope is reformed. Thus, each daughter cell is identical to the cell that underwent mitosis.

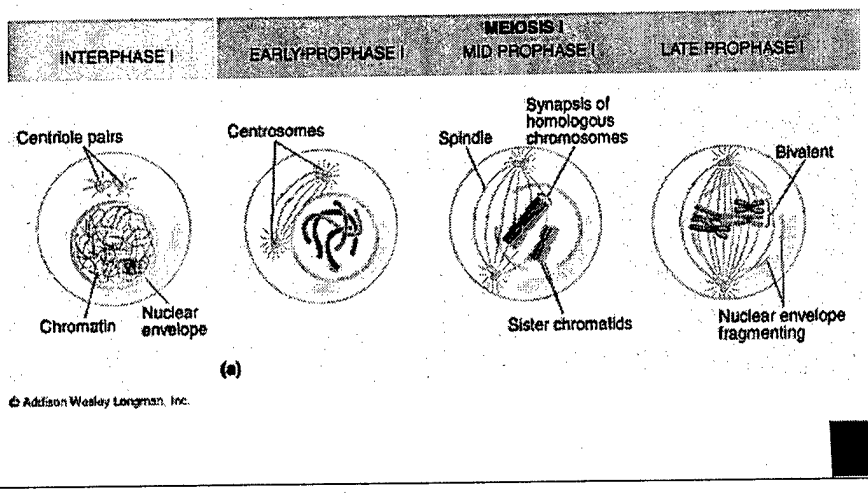
The Cell Cycle - Meiosis



http://genetics.gsk.com/graphics/meiosis-big.gif

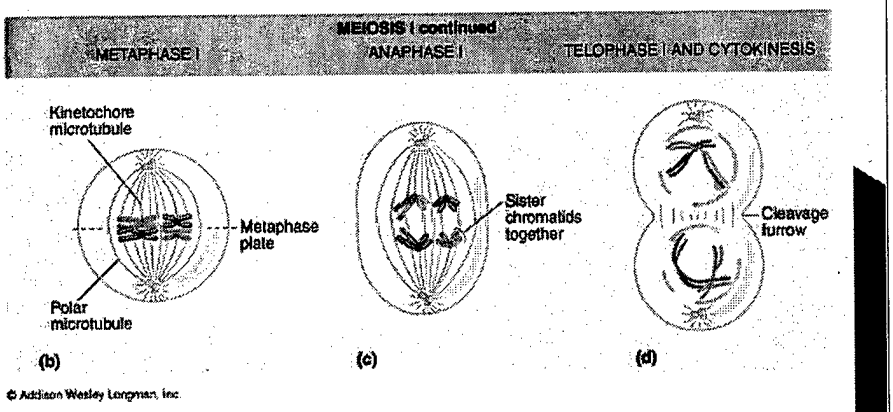
Meiosis is a form of cell division that resembles mitosis; however it involves 2 divisions rather than 1. The result is 4 haploid cells from the cell that undergoes meiosis (either 4 sperm or 1 egg and 3 polar bodies). Meiosis results in haploid gametes, which are called germ cells. Unlike in mitosis, the haploid gametes formed by meiosis are not identical to one another due to the separation of the members of each pair of chromosomes and a process called recombination. During recombination, crossing over occurs between the two parental chromosomes in each chromosome pair undergoing meiosis. The number of chromosomes in the daughters cells is half that of the parent cell. In other words, the cells that are made through meiosis have 23 chromosomes (1 of each chromosome) rather than 46 chromosomes (2 of each chromosome).

Meiosis I - Interphase and Prophase

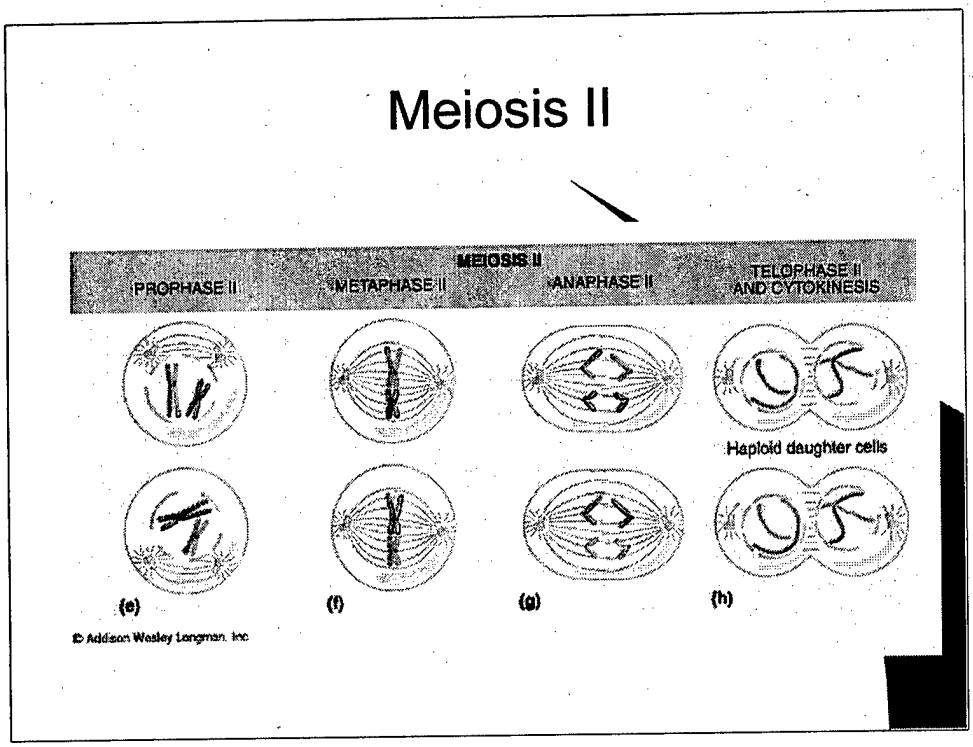


This diagram illustrates the stages of meiosis in an animal cell.

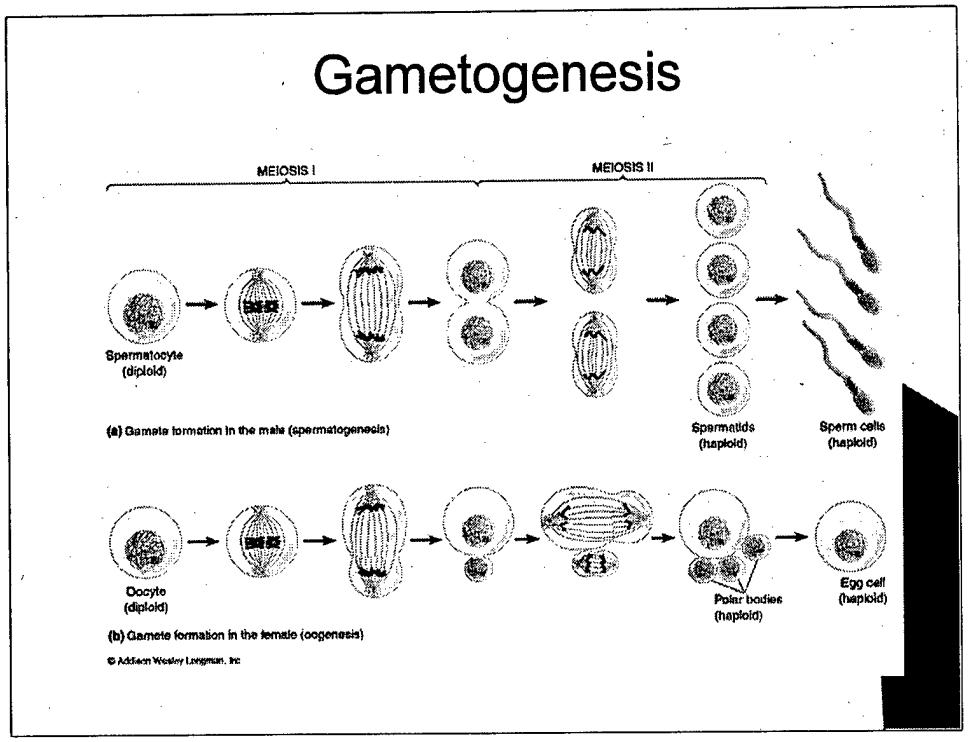
Meiosis I - Metaphase, Anaphase, Telophase, and Cytokinesis



This diagram illustrates the stages of meiosis in an animal cell.



This diagram illustrates the stages of meiosis in an animal cell.



This diagram depicts the process of gametogenesis in both males and females. animals. In spermatogeneis, a diploid spermatocyte undergoes meiosis to produce four haploid (n) spermatids. These differentiate during spermatogenesis to become mature sperm. In oogenesis, a diploid oocyte undergoes meiosis to produce one haploid egg cell and three polar bodies.

Summary Question

Mitosis and Meiosis differ in that mitosis occurs in _____ cells and results in a _____ cell, while meiosis occurs in _____ cells and results in _____ cell.

A) germ, diploid, somatic haploid
B) somatic, haploid, germ, haploid
C) somatic, diploid, germ, diploid
D) somatic, diploid, germ, haploid

Answer

Mitosis and Meiosis differ in that mitosis occurs in _____ cells and results in a _____ cell, while meiosis occurs in _____ cells and results in _____ cell.

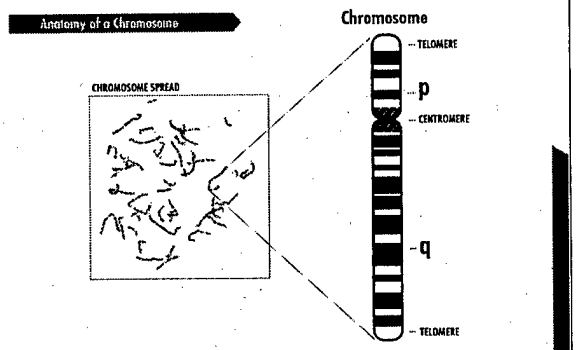
A) germ, diploid, somatic haploid
B) somatic, haploid, germ, haploid
C) somatic, diploid, germ, diploid
D) somatic, diploid, germ, haploid

Lesson 7 – Chromosomes, Normal and Abnormal

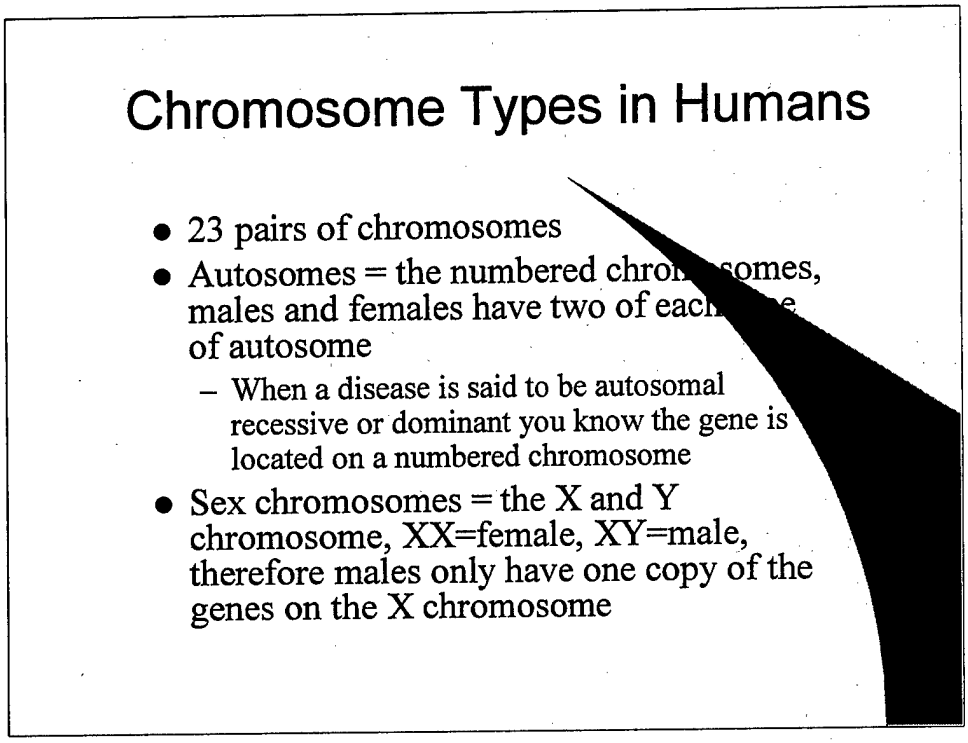
- This lesson discusses the structure of human chromosomes, alleles, genotype/phenotype, karyotypes, as well as numerical and structural chromosomal abnormalities
  - 25 slides

Chromosome Structure

- Stucture
  - Chromosomes are divided into 2 arms that are separated by the centromere:
    - p arm – p for petite
    - q arm
  - Ends of chromosomes are called telomeres



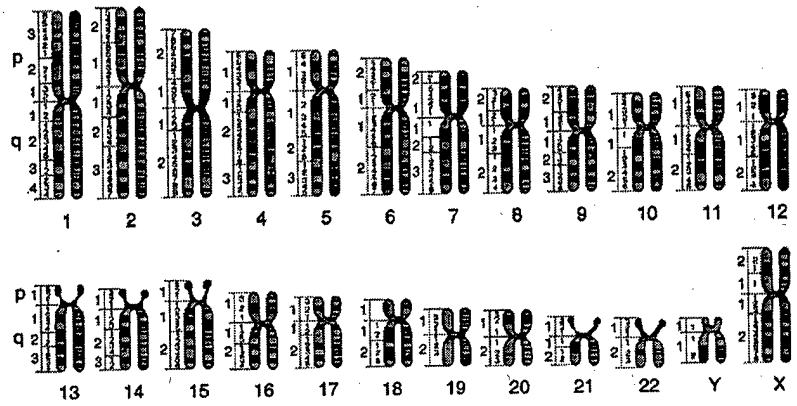
Chromosomes are made up of genes on an extremely long and continuous stretch of DNA. In terms of structure, chromosomes are divided into 2 arms that are separated at the centromere. The short arm is denoted p (for petite) and the long arm is denoted q. The ends of each chromosome are marked by telomeres, which are special DNA regions that play an important role in preserving chromosomal size during cell division.



Humans have 23 pairs of chromosomes in their nucleated cells. Each cell contains 2 copies of each chromosome, so each cell has 46 chromosomes.

In both men and women, the first 22 pairs of chromosomes are called autosomes. Each cell also has 1 pair of sex chromosomes. Females have 2 X sex chromosomes, and males have one X chromosome and one Y sex chromosome.

Features of Normal Chromosomes

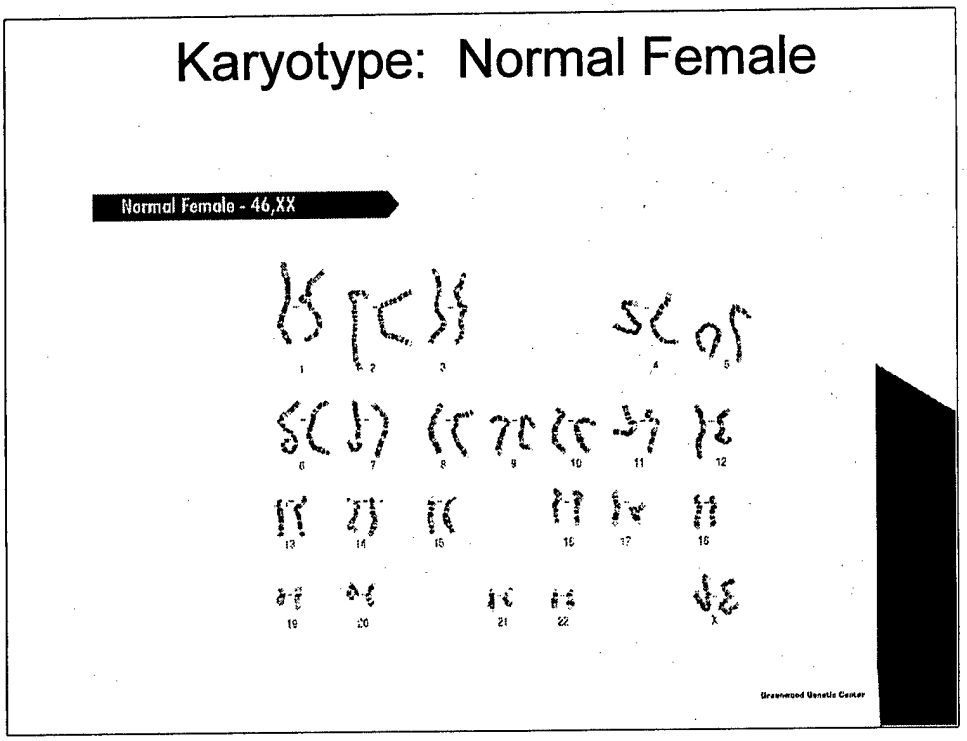


(d) Conventional numbering system of G-bands in human chromosomes

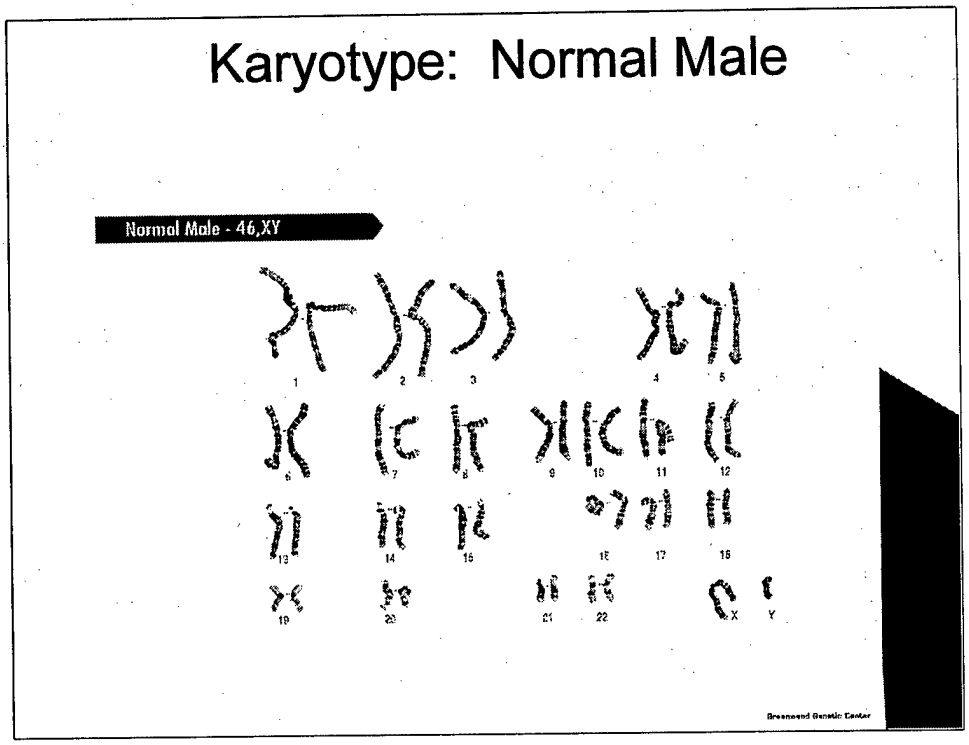
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This figure illustrates the conventional numbering of bands in Giemsa-stained human chromosomes. Giemsa staining is a special process that allows human chromosomes to be distinguished based on the characteristic banding pattern revealed by this process. The banding patterns of chromatids change as the chromatids condense. The left side of each chromosome shows the banding pattern of a chromatid in mid-metaphase, and the right side shows a chromatid as it would appear in prophase.

Karyotype: Normal Female



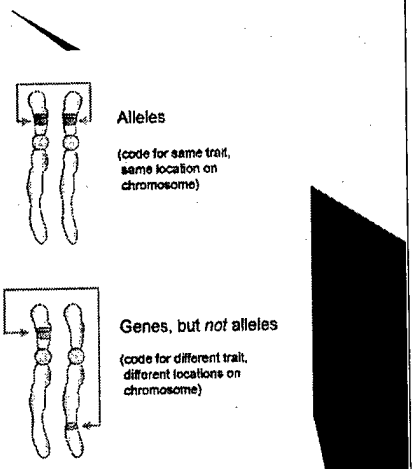
A karyotype is an organized picture of all the chromosomes found in a cell. The chromosomes are arranged from largest to smallest with the sex chromosomes last. A karyotype can show normal chromosomes (46,XX for females and 46,XY for males) or can point out chromosomal abnormalities (such as extra or missing chromosomal material). Therefore, a karyotype can be used to check for structural and numerical changes in chromosomes that may cause genetic disorders. This picture shows a normal female karyotype of 46,XX.



Depicted here is a normal male karyotype of 46,XY.

Alleles

- Alleles = alternate forms of the *same* gene
- One allele is inherited from each parent
- An individual can have 2 identical or 2 different alleles for each gene depending on what allele we receive from our parents

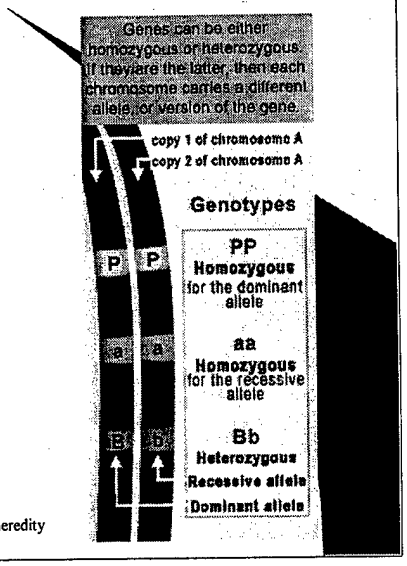


lsvl.la.asu.edu/plb108/ khintze/alleles.jpeg

Members of a pair of autosomes carry matching genetic information. In other words, they contain the same genes in the same location. However, at a given locus (position on a chromosome) a person may have either identical or slightly different forms of the same gene. Alternate forms of the same gene are called alleles. Whether we have two identical or two different alleles for a given gene is dicatated by which alleles we inherit from our mother and father, who both contribute half of their genetic information to their children.

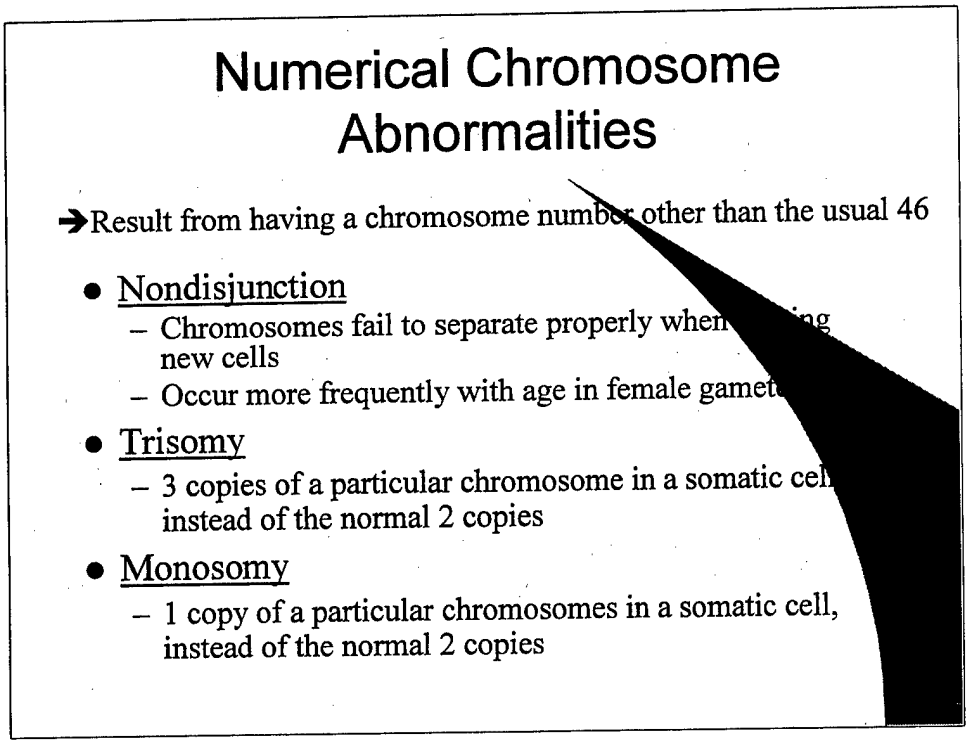
Genotype/Phenotype

- Genotype: the pair of alleles at a particular locus
- Phenotype: the expression of those alleles
- Wild-type: the most common form of a gene
- Homozygous: same allele
- Heterozygous: different alleles



http://genetics.gsk.com/chromosomes.htm#heredity

A person's genotype is defined as the pair of alleles they have at a specific location. In contrast, the term phenotype refers the observable manifestation of a genotype as a trait at the morphological, clinical, or biochemical level. Many genes occur in a single prevailing form called a wild-type allele, as well as in other less common forms. An individual with two identical alleles at a particular locus is homozygous for that gene, whereas a person who has two different alleles at that locus is said to be heterozygous.

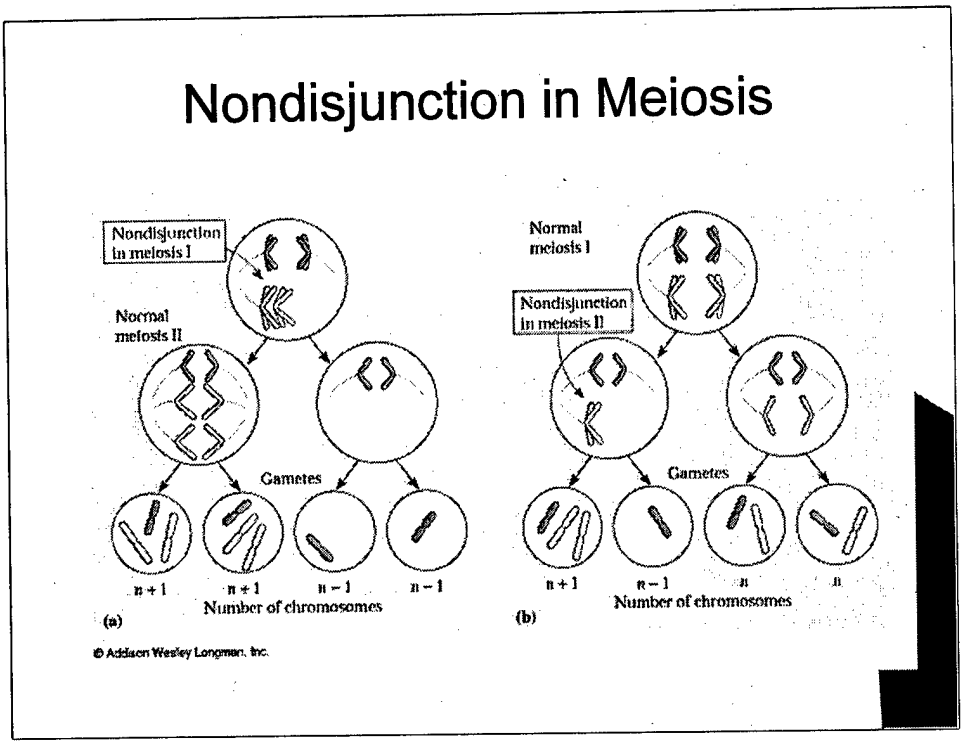


Chromosome abnormalities can be numerical or structural in origin. Numerical abnormalities indicate that there are missing or extra copies of a chromosome or chromosomes.

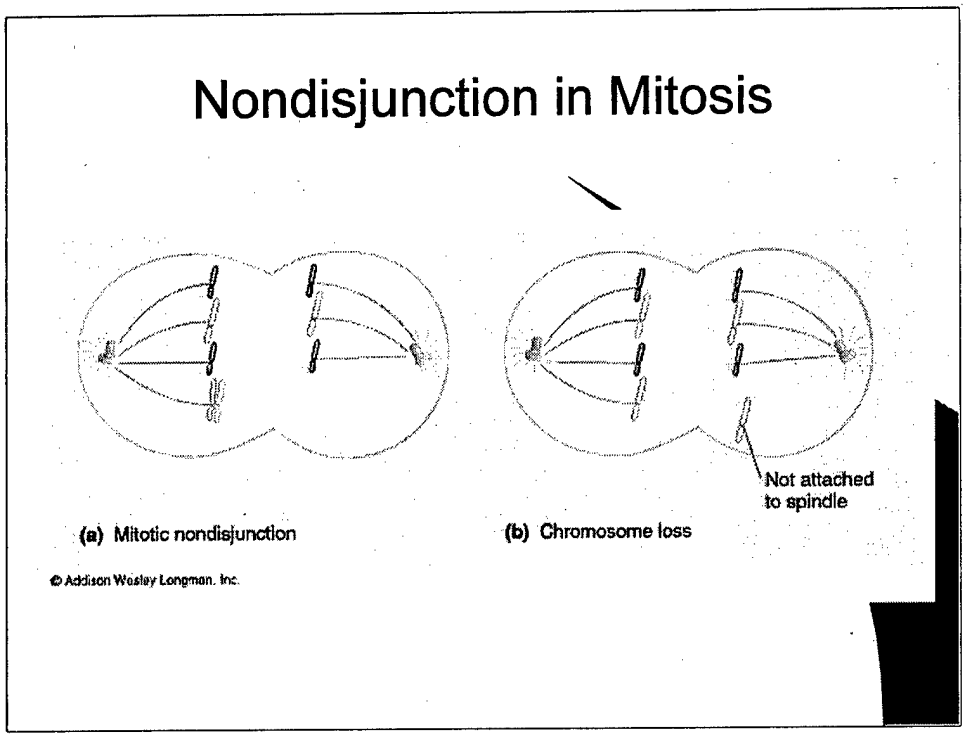
The major cause of numerical chromosome abnormalities is a mechanism called nondisjunction. Nondisjunction is a problem that occurs when chromosomes fail to separate properly during the process of cell division. Although nondisjunction more frequently occurs during meiosis, it can also occur during mitosis. Of note, the risk for nondisjunction in female gametes increases with increasing maternal age.

Trisomy is when a chromosome is represented three times in a somatic cell, instead of the normal two times. Trisomy is different from triploidy, which describes the situation in which all of the chromosomes are represented three times instead of twice.

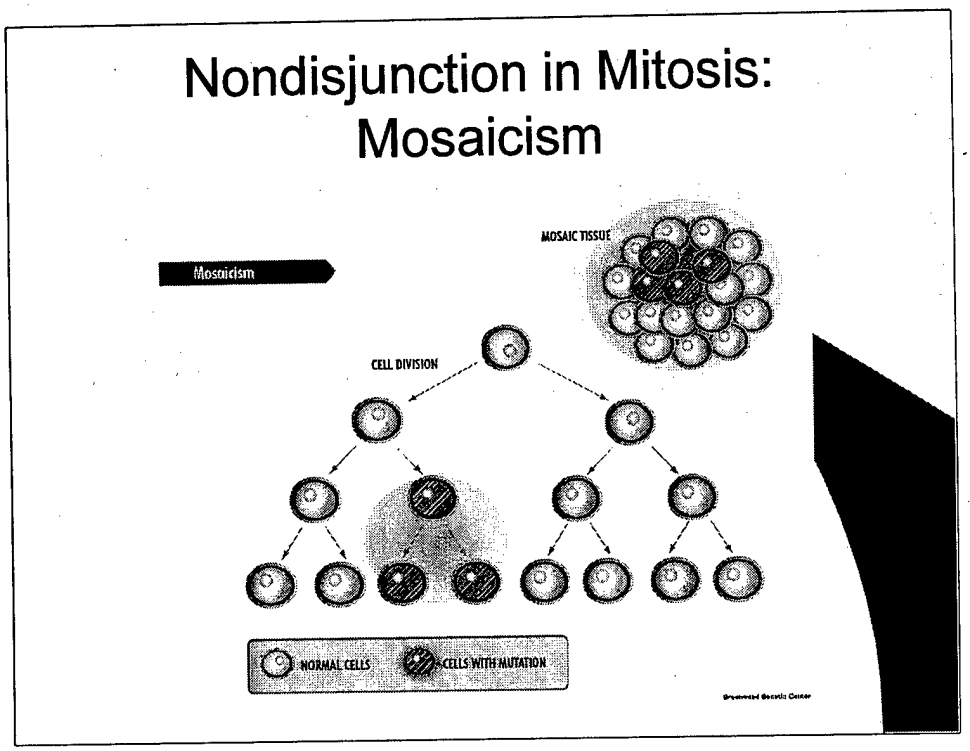
Monosomy is when a chromosome is represented one time in a somatic cell, instead of the usual two times



This diagram shows how nondisjunction can occur during meiotic cell divisions. The pair of chromosomes shown in purple are behaving properly during meiosis I and II, so that each gamete receives one copy of this chromosome. The pair of chromosomes shown in orange are not disjoining correctly. In (a), nondisjunction occurred in meiosis I, so that a gamete receives either two copies of the orange chromosome or zero copies. In (b), nondisjunction occurred during meiosis II, so that one gamete has two orange chromosomes and another gamete has zero. The remaining two gametes are normal.

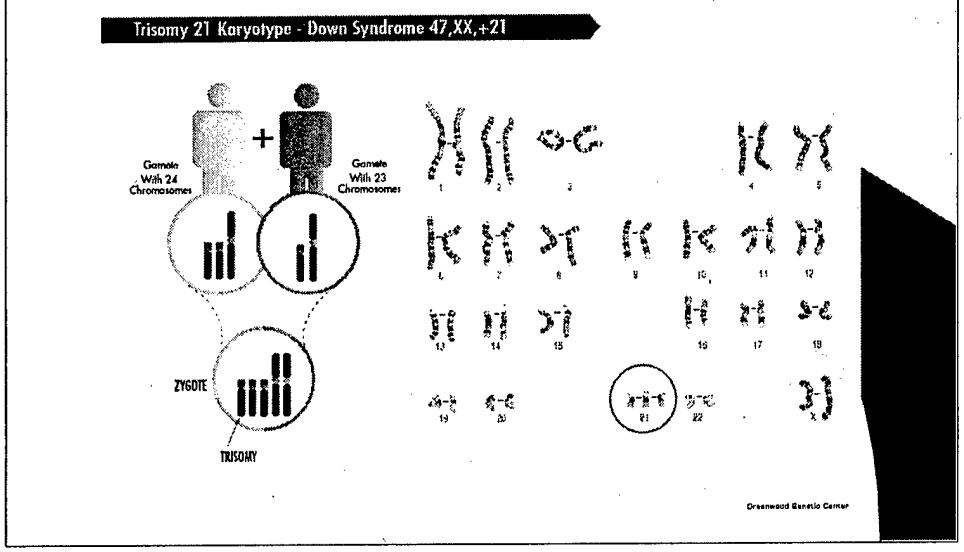


As mentioned previously, nondisjunction can also happen during mitosis, but this is a much less common occurrence. This figure illustrates both nondisjunction (a) and chromosome loss (b) during mitosis in somatic cells. (a) Mitotic nondisjunction produces a trisomic and a monosomic daughter cell. (b) Chromosome loss produces a normal and a monosomic daughter cell.

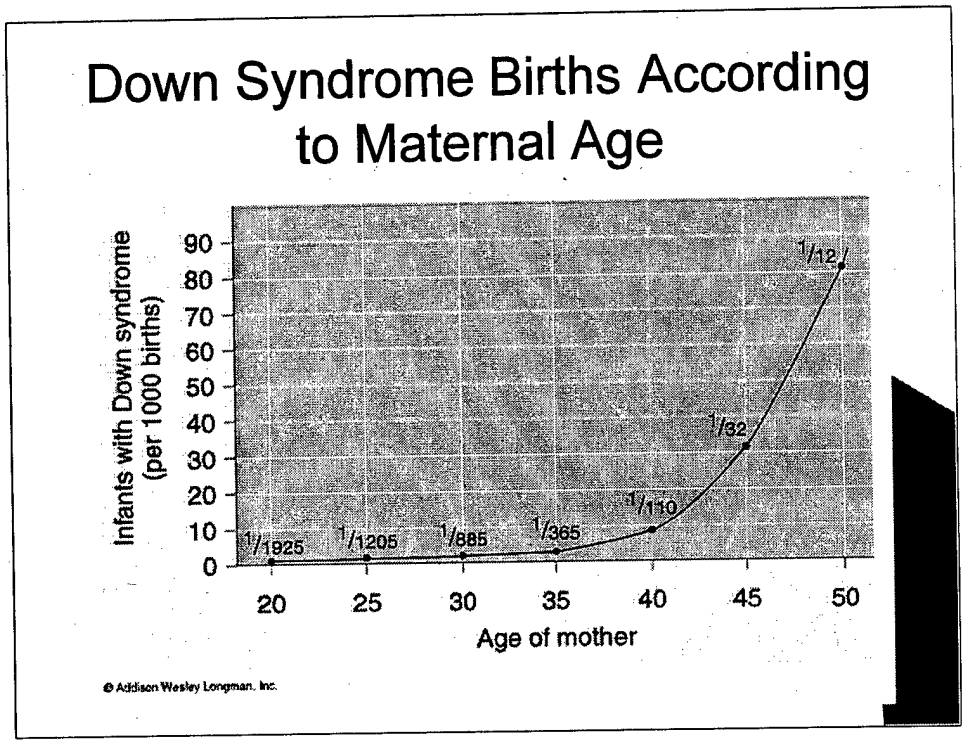


The chromosome abnormalities discussed so far usually result from abnormalities during gamete production (meiosis). However, when nondisjunction occurs in somatic cells, it can lead to often times lead to mosaicism. Mosaicism is a situation in which an individual has at least two cell lines, differing in genotype or karyotype, that are derived from the same zygote. After nondisjunction, some cells can be normal while one or more go awry. In this way, nondisjunction can lead to a mixture of cells, some with the normal number of chromosomes and others with an abnormal number of chromosomes. For instance, a zygote with extra copy of chromosome 21 may lose the extra chromosome during a mitotic division and go on the to develop as a mosaic having cells with the normal chromosome complement, as well as those with an extra chromosome 21.

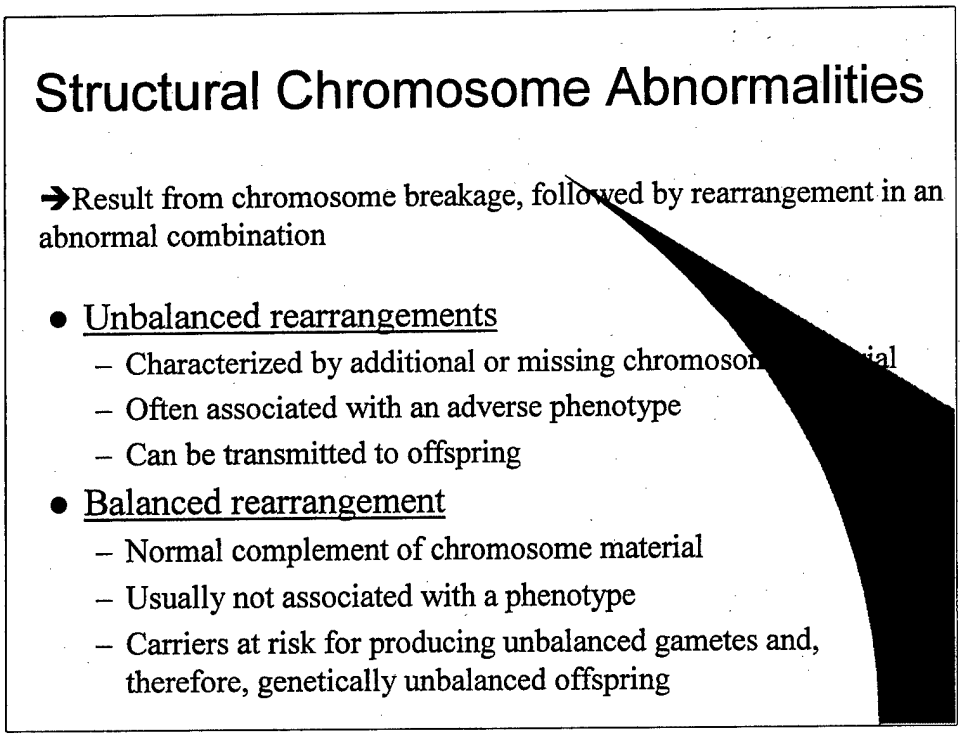
Down Syndrome: Trisomy 21



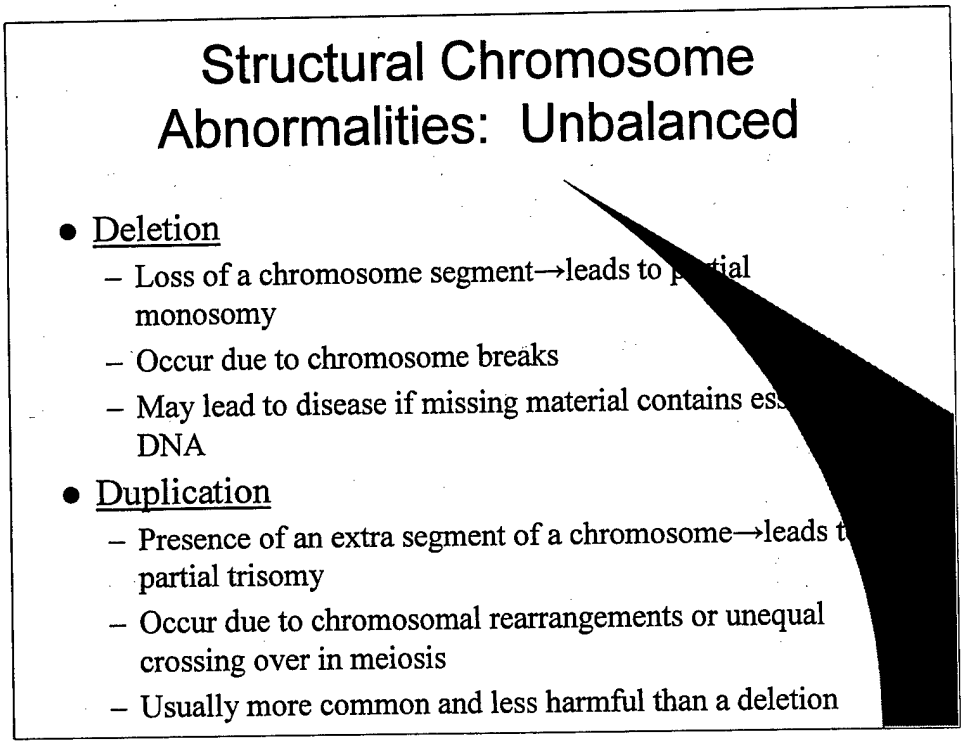
Trisomy for an entire chromosome is usually not compatible with survival. One relatively common exception is trisomy for chromosome 21, which is referred as to Down syndrome. Down syndrome is a type of genetic disorder that occurs in approximately 1 in 800 live births. The majority of Down syndrome cases are caused by nondisjunction. Individuals with Down syndrome have a characteristic physical appearance, often marked by short stature, a flat facial profile, low-set ears, upslanted palpebral fissures, epicanthal folds, a single palmar crease, and mild microcephaly. Other frequently observed features include mental retardation ranging from mild to severe (100%), neonatal hypotonia (~80%), an increased nuchal fold on prenatal ultrasound (~80%), congenital heart disease (~40%), and an increased risk for digestive complications and infections. In addition, about 1% of Down syndrome patients develop childhood leukemia and close to 100% of those over 40 have senile plaques and neurofibrillary tangles, hallmarks of Alzheimer disease. The APP gene, when mutated, is known to cause Alzheimer disease in some families and is located on chromosome 21. .



Nondisjunction of a chromosome is usually a random event that occurs independently in one cell. The risk of nondisjunction occurring in future conceptions is therefore low, usually 1% recurrence risk is quoted. However, maternal age needs to be taken into account also. This is because the risk of having a child with a chromosome problem increases in parallel with maternal age. This graph highlights that the rate of Down syndrome births is dramatically higher among women over the age of 35.

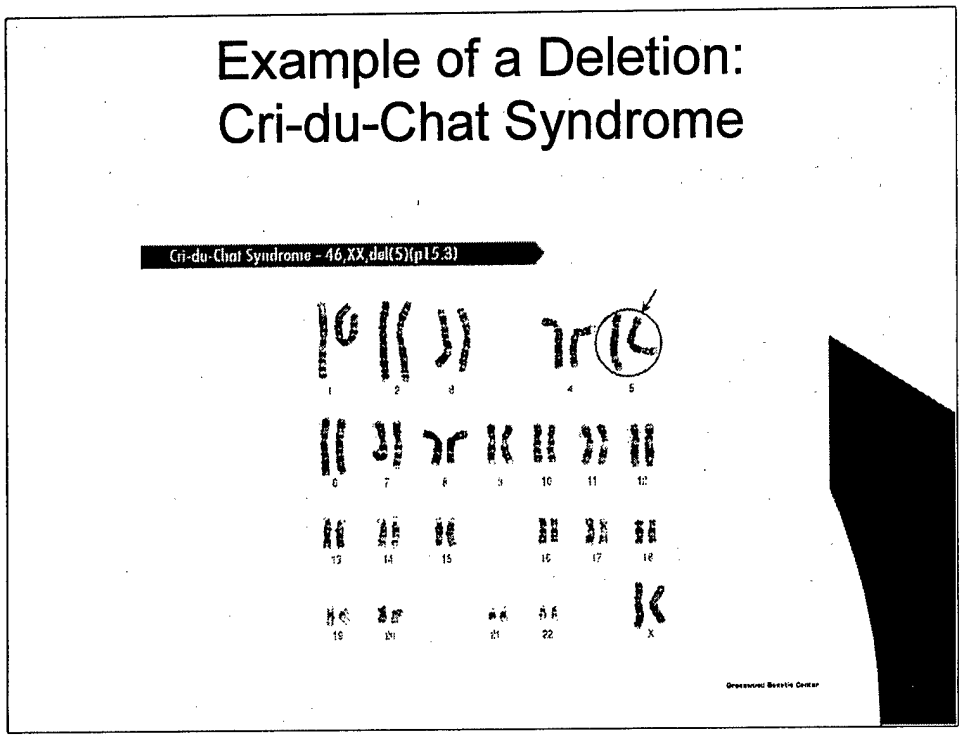


Structural chromosome abnormalities are defined as those resulting from chromosome breakage, followed by rearrangement of the chromosome material in an abnormal fashion. Structural abnormalities can be classified as either balanced or unbalanced depending on the resulting chromosomal constitution. Unbalanced rearrangements are characterized by additional or missing chromosome material. Because the loss or gain of chromosome material can disrupt the normal balance of functional genes, unbalanced rearragements are typically associated with an adverse phenotype. They can also be transmitted to offspring in a simple Mendelian manner. In contrast, structural chromosome problems are called balanced if, after rearrangement, there is still the normal complement of chromosome material. In other words, the chromosome material is all there, it is just packaged differently. These types of rearrangement are not usually associated with a phenotype; however, carriers are at risk for producing unbalanced gametes and, therefore, genetically unbalanced offspring.

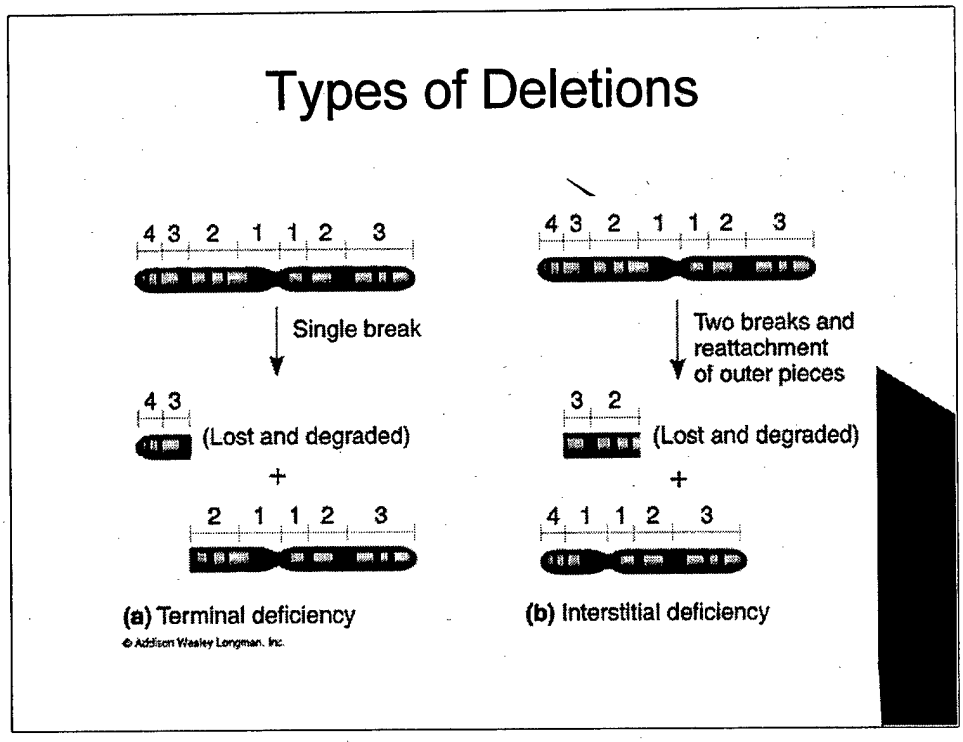


There are a number of different types of structural chromosome abnormalities. The most common of these are deletions and duplications, which lead to partial monosomy and partial trisomy respectively. A deletion is the loss of a chromosome segment of any length, resulting from a chromosome breakage event. The clinical consequence of a deletion depends on whether the missing segment contains a gene or portion of DNA that are essential for normal development. If a critical gene or genes are deleted, a deletion may lead to disease. A duplication is the presence of an extra chromosomal segment that originates from a chromosomal regarrangement or unequal crossing over during meiosis. Generally speaking, duplications are more common than deletions and less harmful, although they can still lead to an abnormal phenotype due to a genetic imbalance.

Example of a Deletion: Cri-du-Chat Syndrome

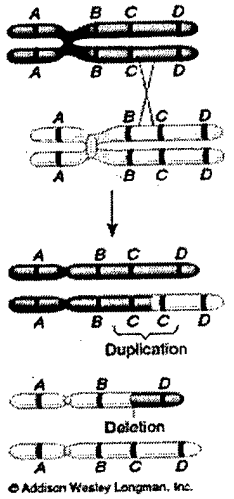


An example of a deletion disorder is Cri-du-Chat syndrome, which is caused by a deletion of a portion of chromosome 5. This specific deletion results in a severe neurologic condition. In terms of deletions, what material is deleted dictates the genes that are missing and, as a result, the condition. This explains why different deletions will have different clinical presentations.

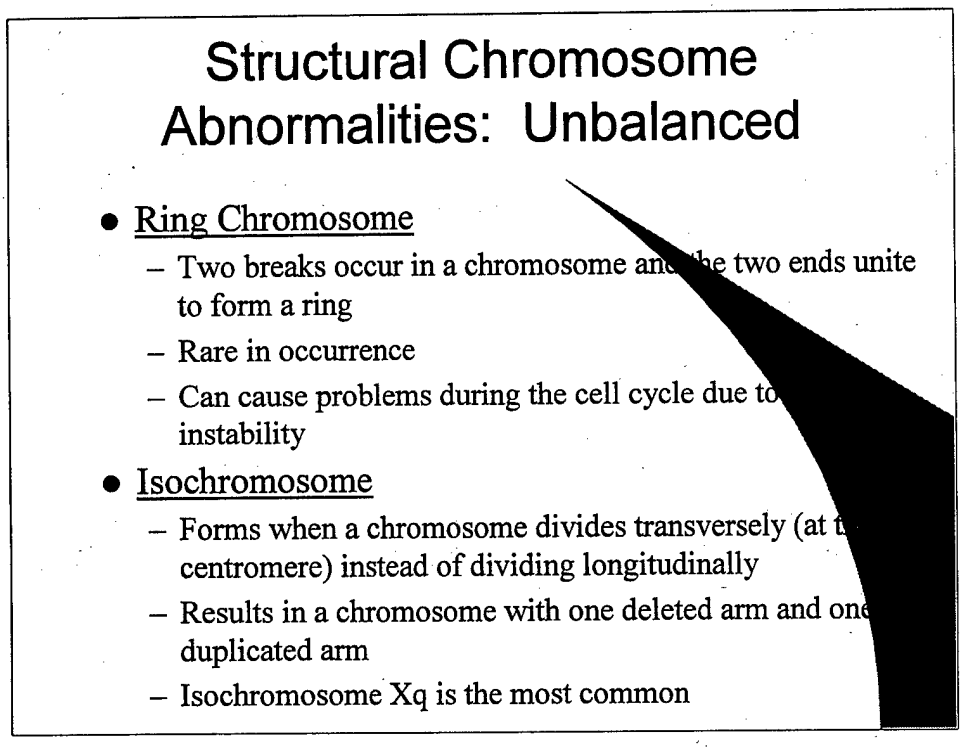


This illustration shows the production of deletions in human chromosomes. There are several different types of deletions that can occur. Terminal deletions are on the ends of chromosomes and usually involve one break. Interstitial deletions occur within a chromosome and usually involve two breaks and the rejoining of the chromosome.

Abnormal Crossing Over Leading to Duplicaton and Deficiency



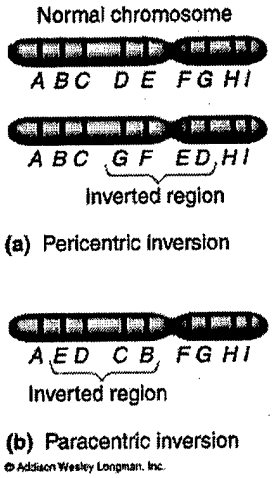
This diagram reveals the mechanism by which abnormal crossing over during meiosis can actually result in both duplication and deficiency. A crossover has occurred at sites between genes C and D in one chromatid and between B and C in another chromatid. After crossing over is completed, one chromatid contains a duplication and the other contains a deletion.



Two other less common types of unbalanced structural rearrangements are ring chromosomes and isochromosomes. A ring chromosome is a special type of deletion in which both ends of a chromosome are lost and the two broken ends unite to form a ring. Because this type of abnormal chromosome is often unstable, it encounters problems during the chromosome replication and distribution phases of the cell cycle. An isochromosome results when a chromosome divides transversely at the centromere instead of dividing longitudinally as it should. The consequence is a duplication of the short arm and the deletion of the long arm, or duplication of the long arm and deletion of the short arm. A person with 46 chromosomes carrying an isochromosome has a single copy of the genetic material of one arm and three copies of the genetic material of the other arm. Ring chromosomes and isochromosomes have been observed in abnormal cells, such as cancer cells.

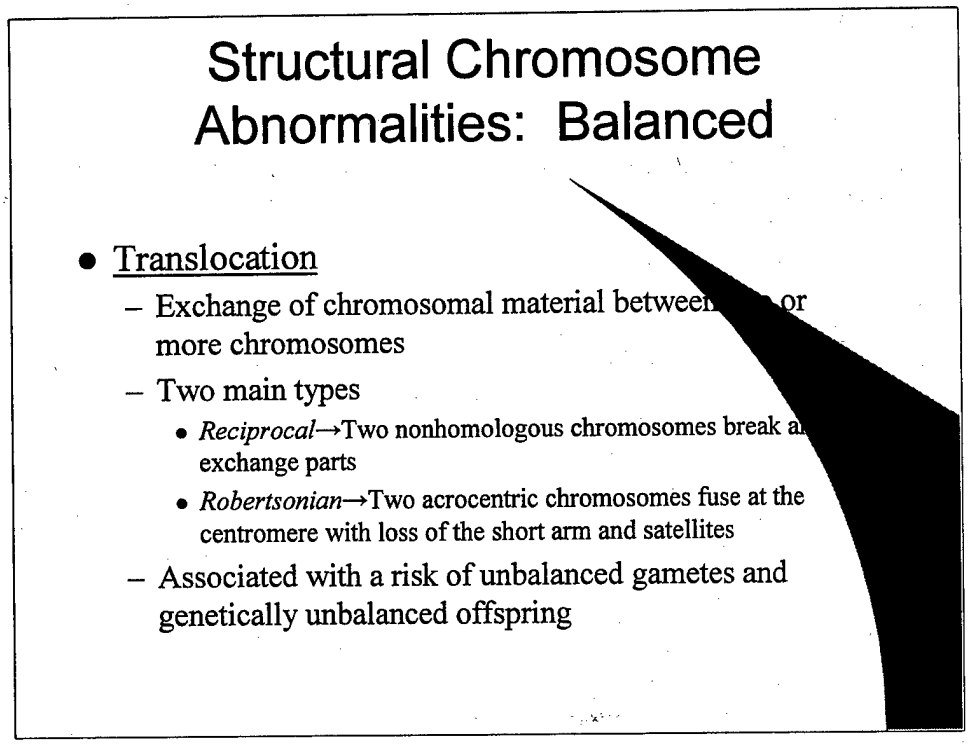
Structural Chromosome Abnormalities: Balanced

- Inversion
  - Two breaks in a chromosome, rotation of that fragment, and reinsertion into the chromosome
  - Does not result in gain or loss of DNA, so no pathology is expected
  - Two types
    - *Pericentric*→includes the centromere
    - *Paracentric*→does not include the centromere

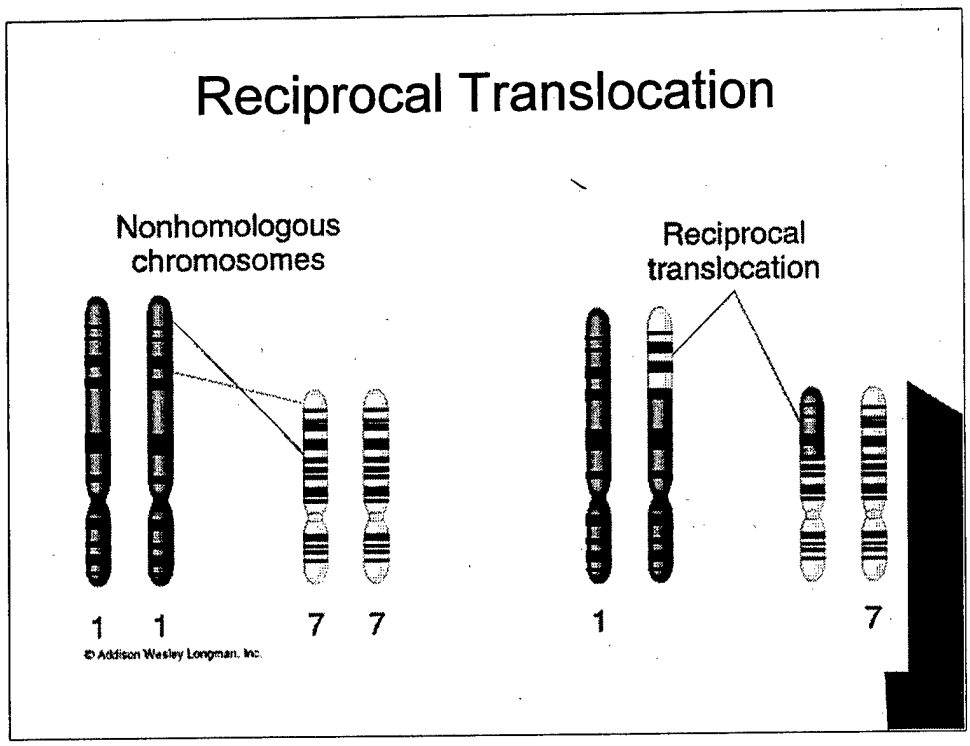


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Just like there are a variety of unbalanced structural rearrangements, there are also several different types of balanced structural chromosome abnormalities. An inversion involves two breaks in a single chromosome, rotation of that fragment, and reinsertion into the chromosome. Two types of inversions can occur: pericentric and paracentric. In a pericentric inversion, there is a break in each arm of the chromosome and, therefore, the centromere is included. However, a paracentric inversion is when both breaks occur in one arm and does not involve the centromere. Inversions, in general, do not result in the gain or loss of chromosome material, so usually no pathology is expected. But, an inversion can cause an abnormal phenotype in offspring because crossing over between a normal and inverted chromosome can generate unbalanced gametes.

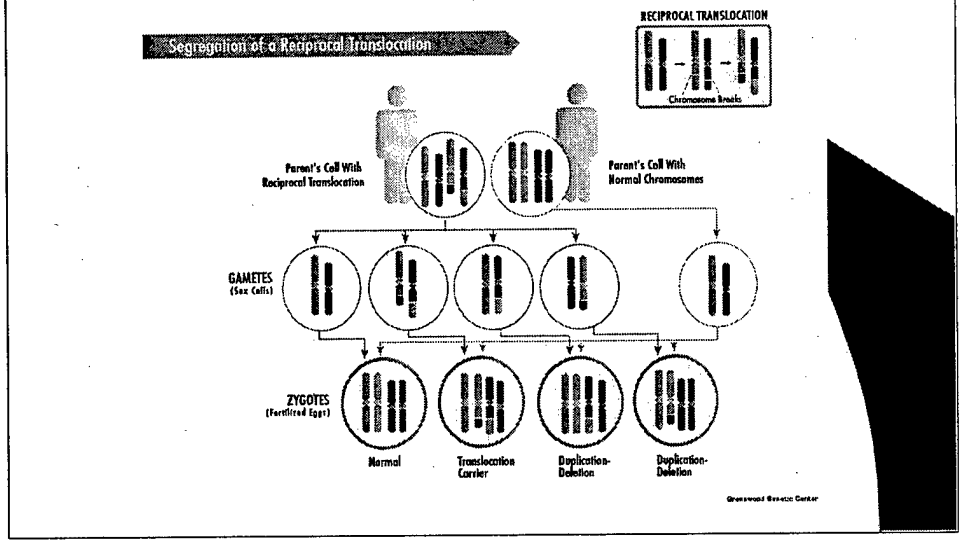


Another type of balanced structural rearrangement is called a translocation. A translocation refers to the exchange of chromosomal material between two or more, usually nonhomologous, chromosomes. There are two main types of translocations: reciprocal and Robertsonian. Although translocation carriers are usually phenotypically normal, they have an increased risk of producing unbalanced gametes and, thus, genetically unbalanced offspring with abnormal phenotypes. A reciprocal translocation occurs when two nonhomologous chromosomes break and exchange their broke-off segements with each other. An acrocentric chromosome is one with the centromere located near one end that has satellited a short arm containing the genes for ribosomal RNA. The acrocentric chromosomes are 13, 14, 15, 21, and 22. A Robertsonian translocation is when two acrocentric chromosomes fuse near the centromere region with loss of their short arms and satellites.



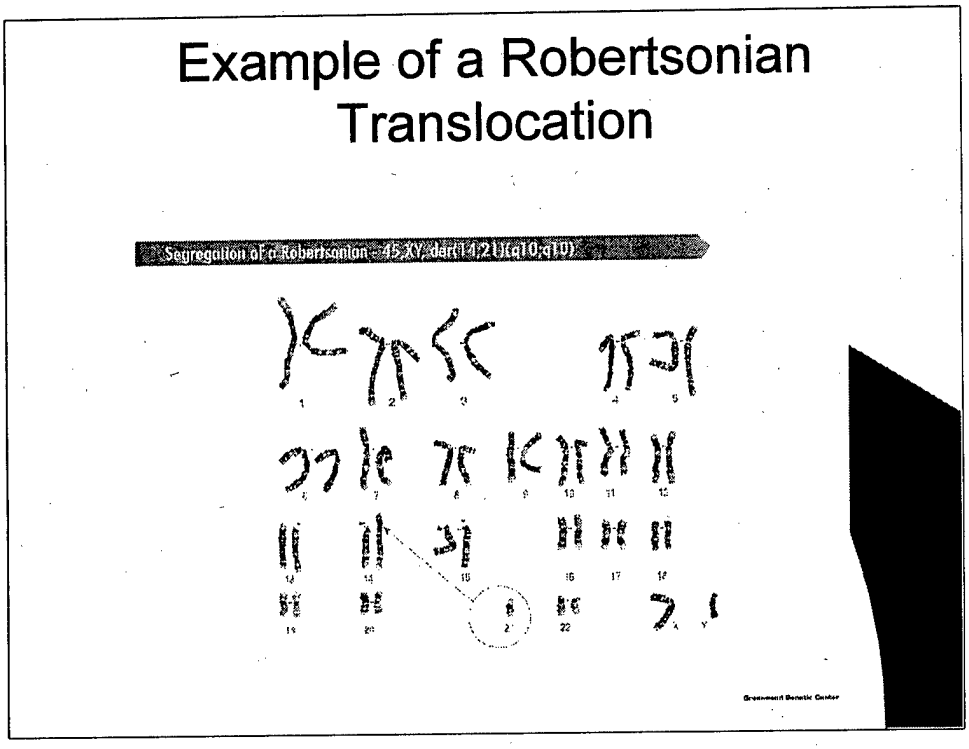
This figure illustrates the formation of a reciprocal translocation. A crossover has occurred between human chromosome 1 and chromosome 7. This crossover yields two chromosomes that carry translocations.

Segregation of a Reciprocal Translocation



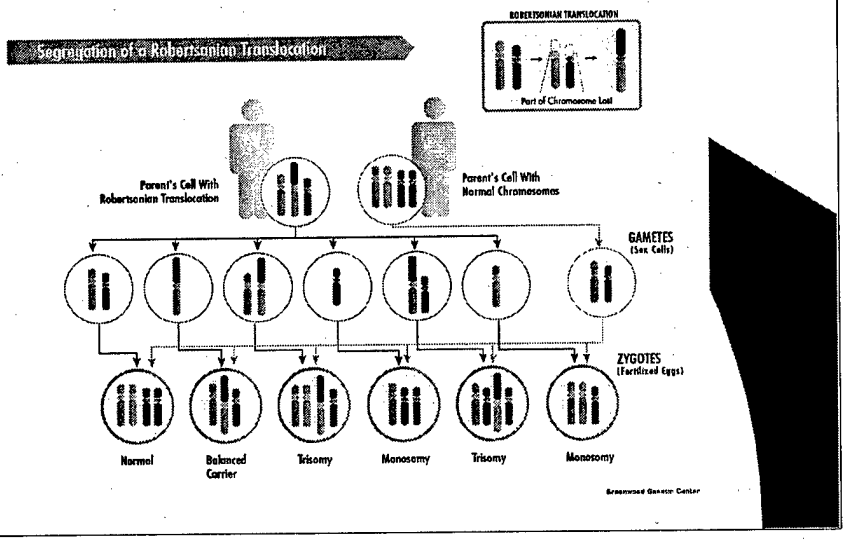
This diagram outlines the segregation of a reciprocal translocation during gametogenesis in a carrier. If no essential chromosomal material is lost and no genes are damaged by the breakage and reunion, a translocation carrier is said to have a **balanced translocation** and, therefore, will usually show no clinically evident phenotype. However, this individual has a high risk of producing unbalanced gametes and, consequently, offspring with an abnormal phenotype.

Example of a Robertsonian Translocation



The karyotype shown here is from a balanced Robertsonian translocation carrier. Specifically, this individual carries a Robertsonian translocation between chromosomes 14 and 21, which is a relatively common type of Robertsonian translocation. It is interesting to note that his karyotype has only 45 chromosomes, yet there is no missing or extra genetic material-it has just been rearranged.

Segregation of a Robersonian Translocation



This diagram shows how a Robersonian translocation segregates during gametogenesis in a carrier. The loss of the short arm of an acrocentric chromosome is not clinically significant because individuals with balanced Balanced carriers of Robertsonian translocations are clinically normal. However, this person is at risk for producing unbalanced gametes, and therefore genetically unbalanced offspring, just like someone who is a carrier of a reciprocal translocation.

Summary Questions

Chromosomes that are numbered and are not involved in sex determination are called:

A) Loci
B) Autosomes
C) Sister Chromatids
D) Hemizygous

When a pair of chromosomes fail to separate during cell division and travel together into the same cell this is called:

A) Nondisjunction
B) Disjunction
C) Meiotic failure
D) Incomplete penetrance

Answers

Chromosomes that are numbered and are not involved in sex determination are called:

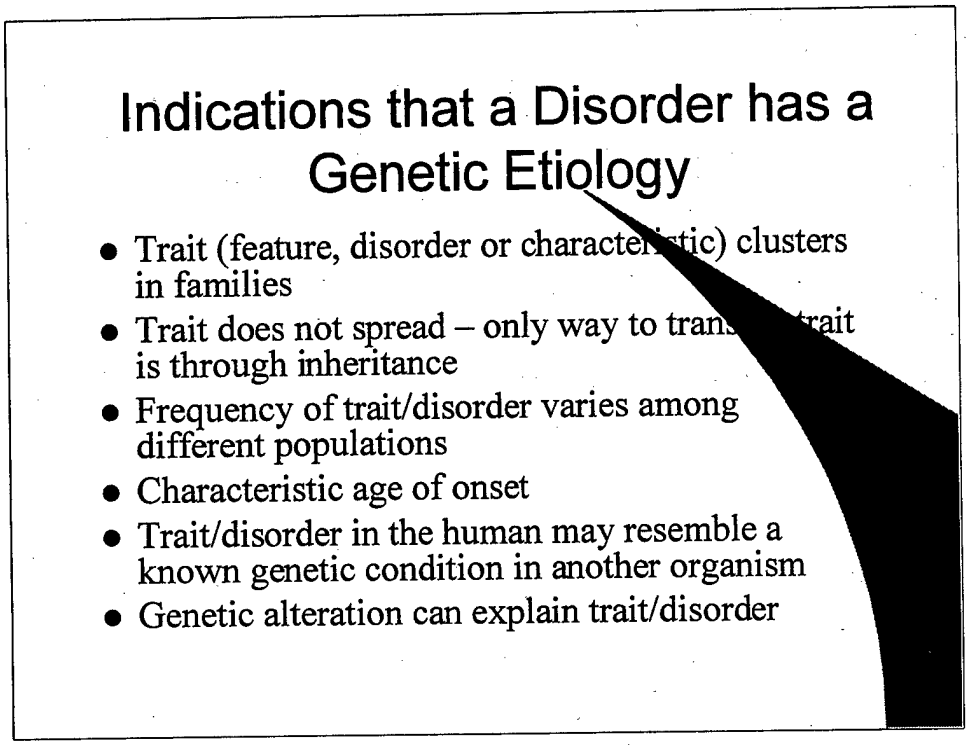
A) Loci
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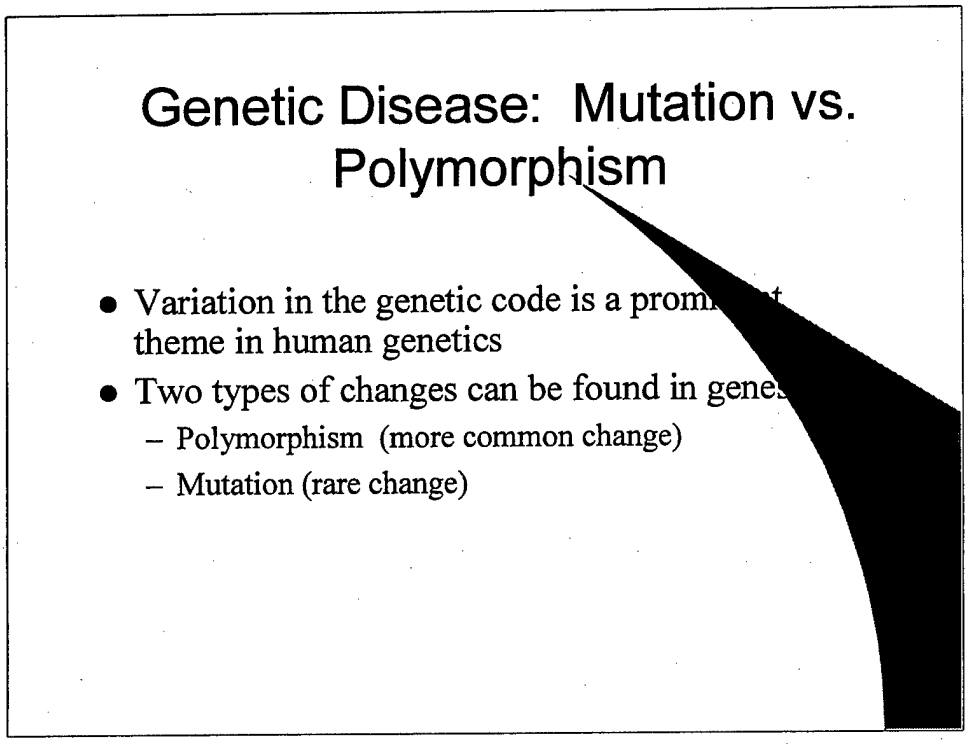
A) Nondisjunction
B) Disjunction
C) Meiotic failure
D) Incomplete penetrance

Lesson 8 – Mendelian Genetics

- This lesson covers what constitutes a genetic disease, the different types of genetic variation, how a family history can provide valuable information about genetic diseases, and Mendelian inheritance patterns
  - 7 slides



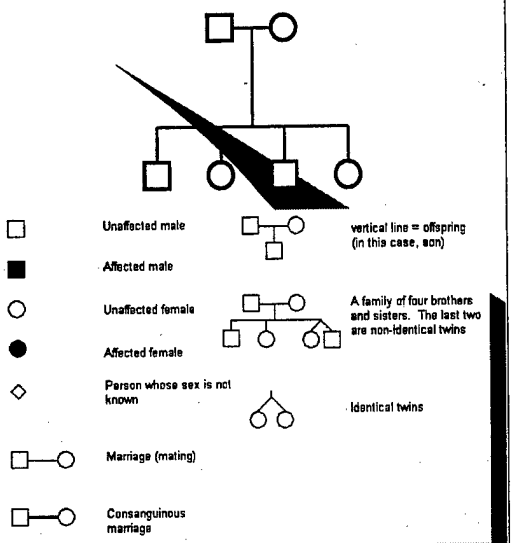
These key characteristics of a disorder are suggestive of an underlying genetic basis. However, a disorder does not have to display all of these features in order to be considered a genetic disease. In fact, many genetic diseases have a very heterogeneous clinical presentation and, therefore, may not have a consistent age of onset in all affects families or individuals.



Inherited variation in the genetic code is the cornerstone of human and medical genetics. Variations in genes can be thought of in two categories: polymorphism and mutation. A polymorphism is a variation in a gene that is at a frequency of >1% in the population. An example of a polymorphism is Rh factor status (Rh+ or Rh-). A mutation is a variation in a gene that occurs at a frequency of <1% in the population. For example, Huntington disease is caused by a mutation in one allele of the huntington gene, which would function normally if not for the mutation.

Genetic Disease: Family History

- Genetic diseases are often familial
- Family histories provide clues about inheritance patterns
- Pedigrees are pictorials displays of a family history

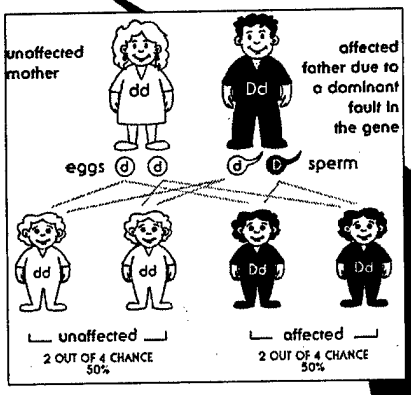


www.ucl.ac.uk/~ucbhjow/ medicine/mendel.html

Genetic diseases often run in families. This occurs because gene mutations can be passed down from one generation to the next. A family history can help elucidate the inheritance pattern of a genetic disorder. Family histories are taken by asking the patient about their relatives and the medical histories of their relatives. It is easy to illustrate the relatedness of a family and what diseases they have by using a chart called a pedigree. Module 3 will expand how to take a detailed family history and draw out a pedigree.

Mendelian Genetics: Autosomal Dominant Inheritance

- Autosomal Dominant
  - Gene located on non-sex chromosome
  - Present in every or almost every generation (vertical transmission)
  - Each affected person has an affected parent
  - Unaffected family members do not transmit the trait
  - Trait can be transmitted by both sexes and both sexes are equally at risk

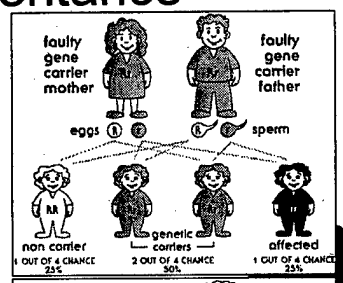


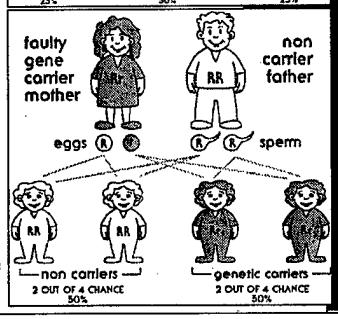
www.genetics.com.au

If a disease is considered autosomal dominant, the disease is present in every or almost every generation. The inheritance patterns seen with an autosomal dominant condition are as follows: 50% of the time the unchanged gene will be passed on and an unaffected child will be the result. The other 50% of the time the changed gene will be passed on resulting in an affected child. For dominant conditions the inheritance patterns hold true no matter which parent is affected and no matter what the sex of the offspring. Examples of diseases that show autosomal dominant inheritance are neurofibromatosis types 1 and 2, achondroplasia (dwarfism), familial hypercholesterolemia, and Huntington disease.

Mendelian Genetics: Autosomal Recessive Inheritance

- Autosomal Recessive
  - Gene located on non-sex chromosome
  - Present usually only in siblings (horizontal transmission)
  - Males and females are equally likely to be affected
  - May appear sporadic



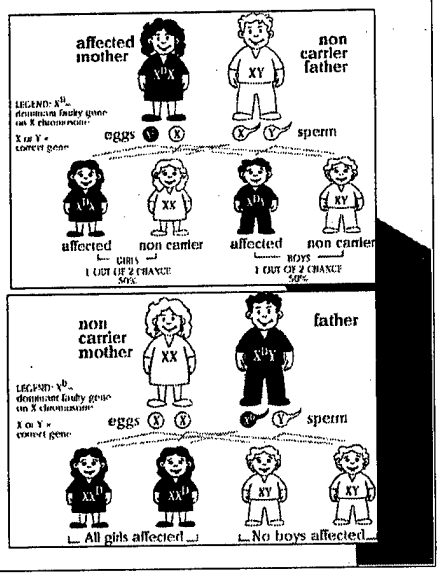


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If a disease is considered autosomal recessive, it will most likely be present in only one or a few individuals. If both parents are carriers, 25% of the time the unchanged gene will be passed on by each parent resulting in an unaffected, non-carrier child. 50% of the time one copy of the unchanged gene and one copy of the changed gene will be passed on resulting in an unaffected carrier child. The other 25% of the time two copies of the changed gene will be passed on (one from each parent) resulting in an affected child. If only one of the parents is a carrier (it does not matter if it is the mother or the father), 50% of the children will be carriers and the other 50% of the children will be non-carriers. All of these children will be unaffected. Examples of autosomal recessive diseases are cystic fibrosis, sickle cell anemia, and β-thalassemia.

Mendelian Genetics: X-linked Dominant Inheritance

- X-linked Dominant
  - Gene located on X chromosome
  - Affected males have no affected sons and no unaffected daughters
  - Affected females are twice as common as affected males for rare diseases

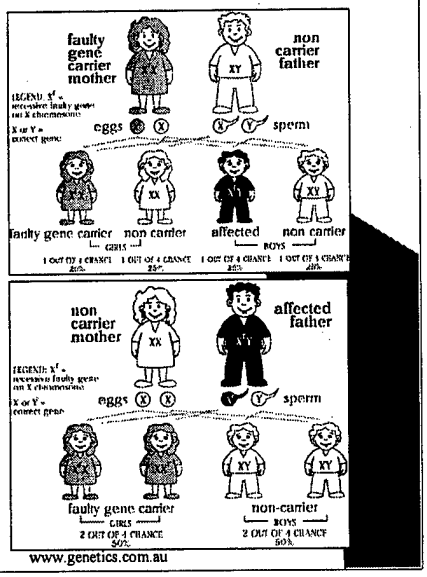


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X-linked dominant conditions occur when only one copy of the changed gene is necessary to cause the individual to be affected. Both males and females are affected in X-linked dominant conditions. If the mother is affected, 50% of her daughters will be affected and 50% of her sons will be affected as can be seen in the first figure on the right. If the father is affected, all of his daughters will be affected and none of his sons will be affected. X-linked dominant conditions are extremely rare. An example of an X-linked dominant disorder is Rett syndrome.

Mendelian Genetics: X-linked Recessive Inheritance

- X-linked recessive
  - Incidence is higher in males than in females
  - Affected males in family are related through unaffected females
  - No male to male transmission
  - Daughters of affected males are obligate carriers



X-linked conditions can also be recessive. These conditions occur when two copies of the changed gene are present or an unchanged gene is not present to mask the changed gene. Usually males are affected by X-linked recessive conditions because they only have one copy of the X chromosome (hemizygous). On the other hand, females have two copies of the X chromosome and can be carriers of the changed gene. If the mother is the carrier of the changed gene, then 50% of her sons will be affected and 50% of her daughters will be unaffected carriers. If the father has the changed gene, he will be affected. He will pass the changed gene to all of his daughters, who will all be unaffected carriers. None of his sons will be affected because the father passes his Y chromosome to his sons. Examples of X-linked recessive conditions are hemophilia A and B and Duchenne muscular dystrophy.

Summary Questions

Two healthy individuals have a son with Krabbe disease (autosomal recessive). They want to know their risk of their next pregnancy resulting in another child with Krabbe disease. What would that risk be?

A) 25%
B) 50%
C) 12.5%
D) 100%

A man has hemophilia (a recessive X-linked condition) and mates with a female who is a known carrier. What are the probabilities for offspring?

A) Half of their daughters and half of their sons will be affected
B) None of their daughters and half of their sons will be affe
C) All of their daughters will be carriers and none of their so be affected
C) All of their daughters and all of their sons will be affected

Answers

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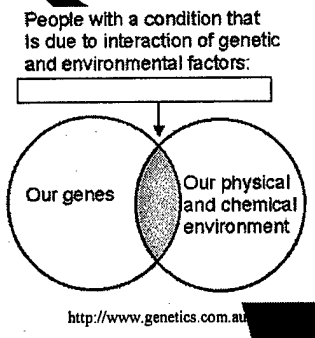
Lesson 9 – Non-Mendelian Genetics

- Mendelian inheritance patterns are relatively simple to pick out of a family history. A challenge is presented when the inherita pattern does not seem to fit any of the pre described patterns. The truth is, most genet diseases do not follow the simple patterns we have discussed thus far. Most genetic diseases can be described as complex or multifactorial and relating risk to families with these disorders can be quite difficult.
  - 9 slides

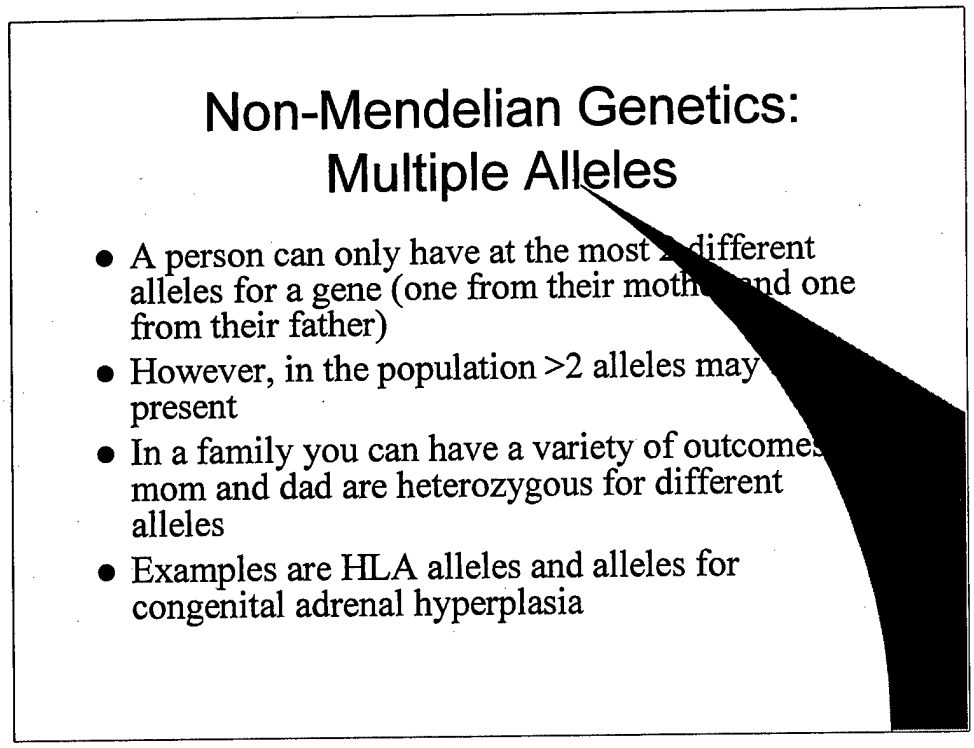
Non-Mendelian Genetics: Complex Disease

- Non-Mendelian genetics refers to inheritance patterns that do not follow Mendel's laws
- Genotype and Environment are responsible for complex or multifactorial diseases
- Genotype conveys a certain amount of susceptibility for or protection against a multifactorial disease

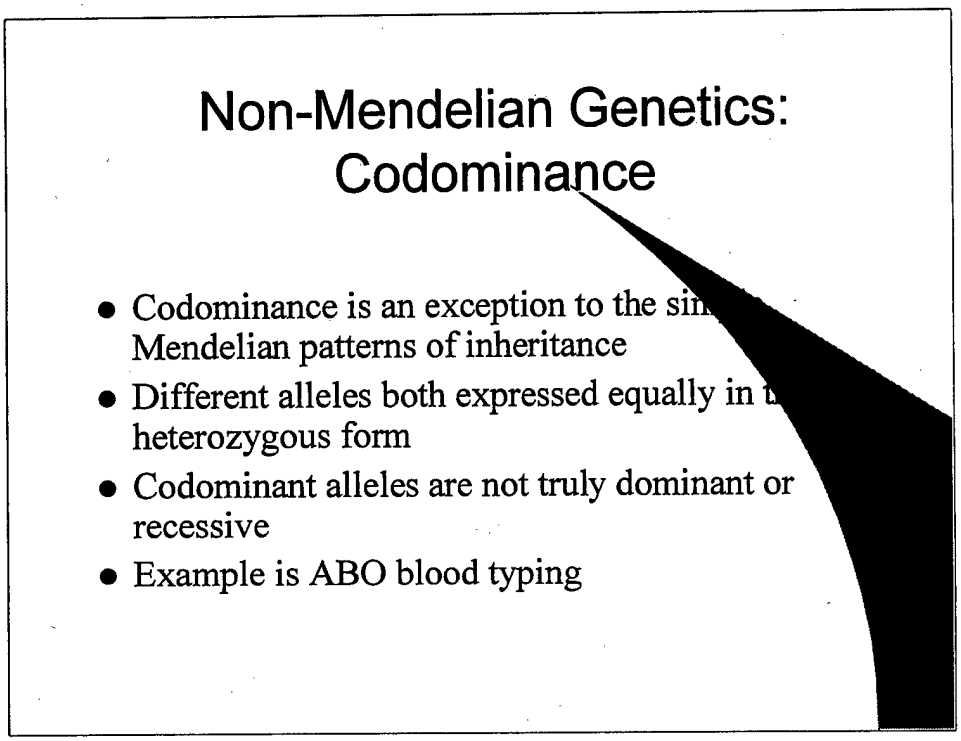
  Examples: diabetes, heart disease, cancer



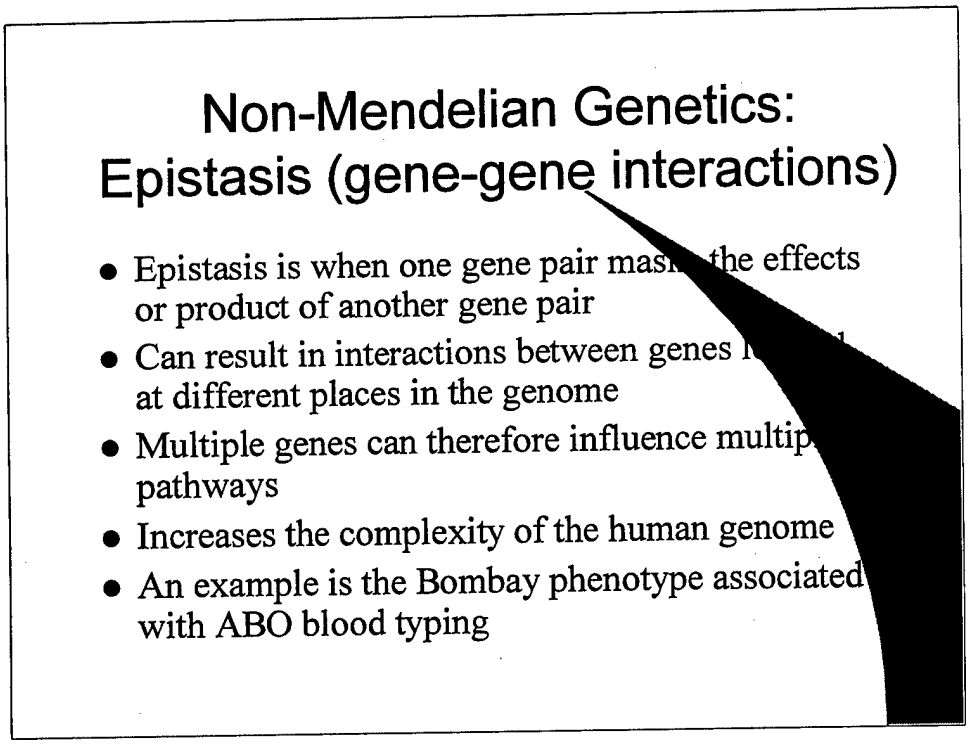
Complex or multifactorial diseases have more than one component contributing to the disease phenotype. These disease result from a combination of one or several genes interacting with stimuli from the environment. The environment affects individuals differently based on their unique genetic makeup. Also, the disease state is variable between individuals due to one person having a different environment than another (i.e. different exposures, different diet, different amounts of exercise, different climate, etc.). Examples of multifactorial diseases are diabetes, heart disease, and cancer. Certain viruses are known to integrate their own genetic material into our DNA and to cause problems which may trigger the onset of a multifactorial disease.



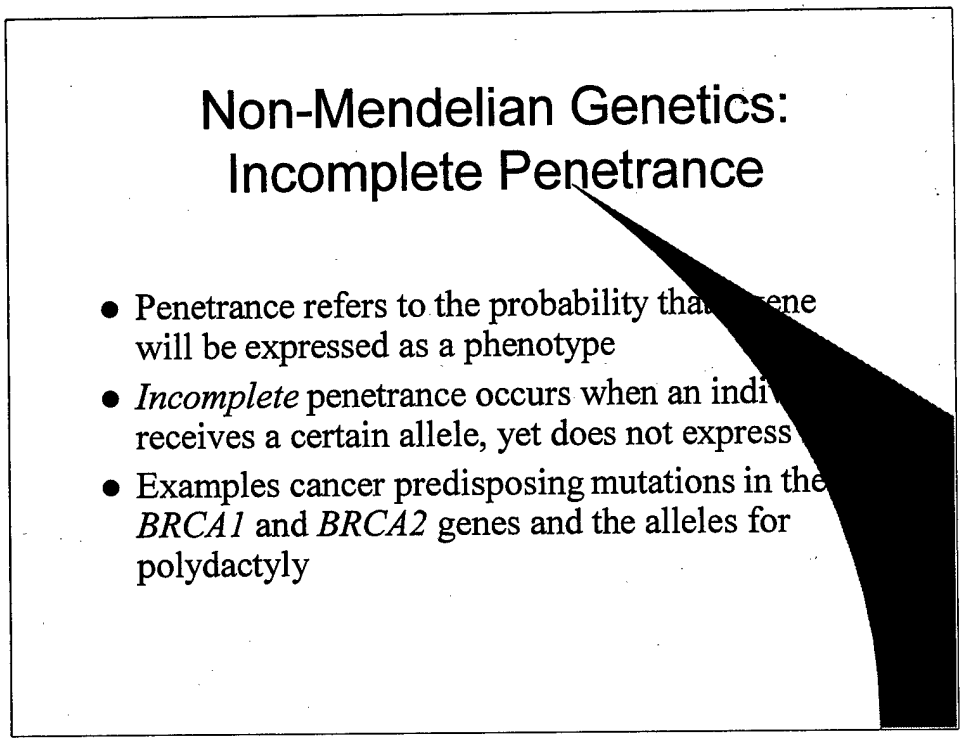
Some traits are controlled by multiple alleles. An example is the HLA gene system, which is responsible for identifying and rejecting foreign substances in the body. This system is comprised of 6 different genes that each have a large number of possible alleles. Amazingly, 30,000,000 different HLA genotypes can occur due to the presence of such as vast number of possible alleles for each of the six HLA genes. Another example of the effect of multiple alleles is congenital adrenal hyperplasia, an inherited disorder of cortisol biosynthesis resulting in virulization of female infants. Four distinct forms of this disorder are found based on what combination of alleles is inherited.



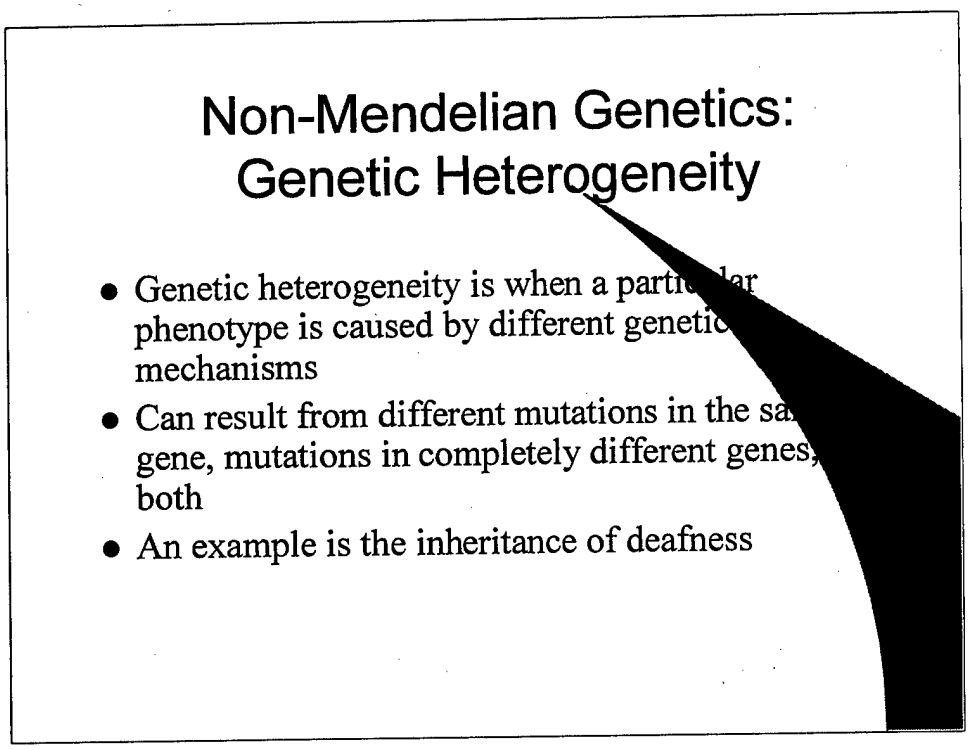
Codominance is an interesting exception to the simple Mendelian patterns of inheritance in which expression of each allele can be detected even in the presence of the other. Therefore, alleles that show codominance are not simply recessive or dominant. This phenomenon is illustrated by the ABO blood typing system. People with the AB blood type have the characteristics of both type A and type B blood and their phenotype is intermediate between the two.



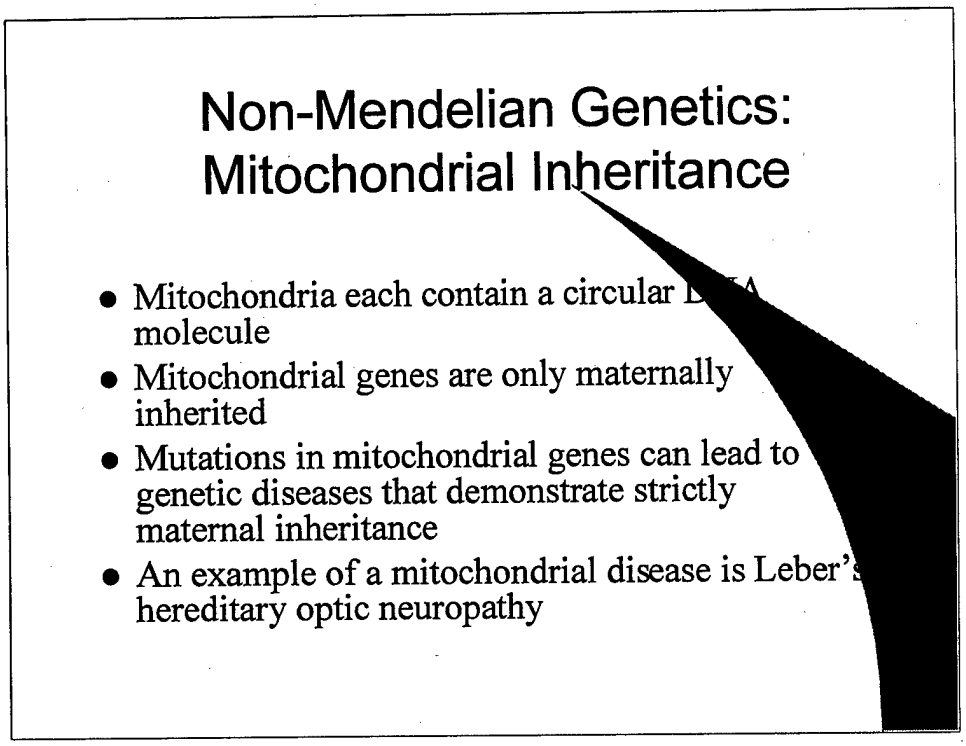
Epistasis results when two or more genes interact to control a single phenotype. In this way, one or more genes can potentially interfere with the expression of another gene and multiple genes can influence multiple pathways in the body. For instance, a mutation preventing an early step in a particular biochemical pathway can prevent other downstream products from being formed. An additional example is the Bombay phenotype. As long as the genotype at this gene is HH or Hh, the ABO alleles determine the ABO blood type. However, if the genotype is hh, then the A or B antigens cannot adhere to the red blood cell and your have an O phenotype even though a person may be AB at the genetic level.



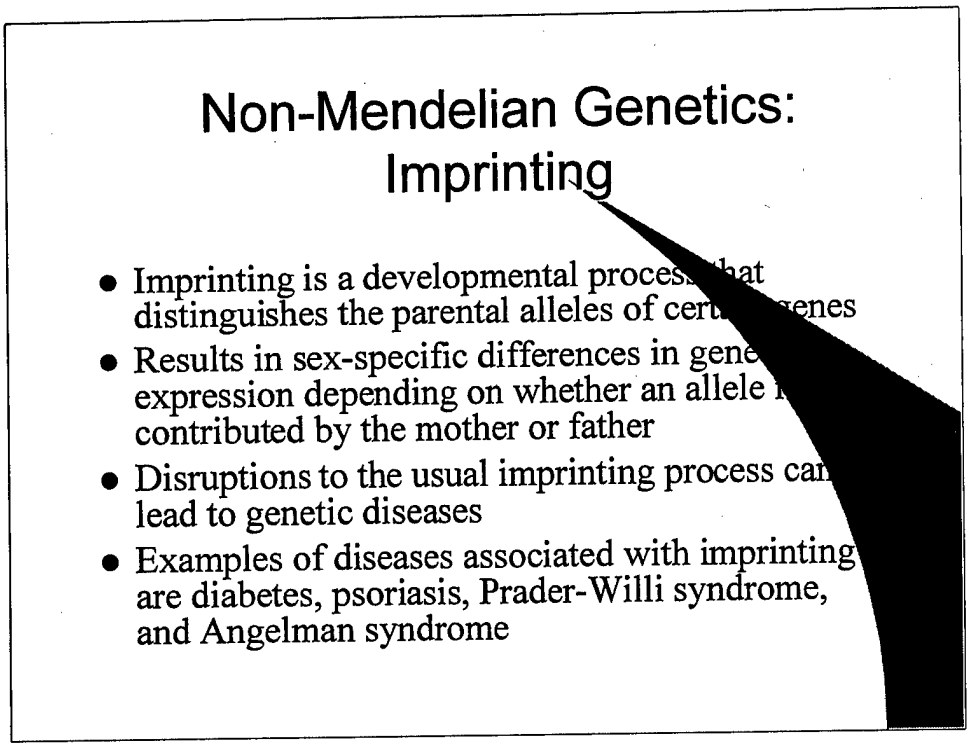
Not all traits are expressed 100% of the time even though the allele is present. When the frequency of expression of a phenotype is less then 100%, the gene is said to show incomplete penetrance. Therefore, penetrance is an all-or-none concept. For example, women with inherited mutations in either their *BRCA1* or *BRCA2* gene have a significantly increased risk for developing breast and/or ovarian cancer at younger ages than in the general population. However, not all women with these mutations will develop these types of cancer in their lifetimes. Another example is polydactyly (extra fingers or toes). The allele for six fingers is a dominant trait and is usually expressed. However, not all people with this allele have six fingers, yet they can still pass it along to their children, who can express the trait.



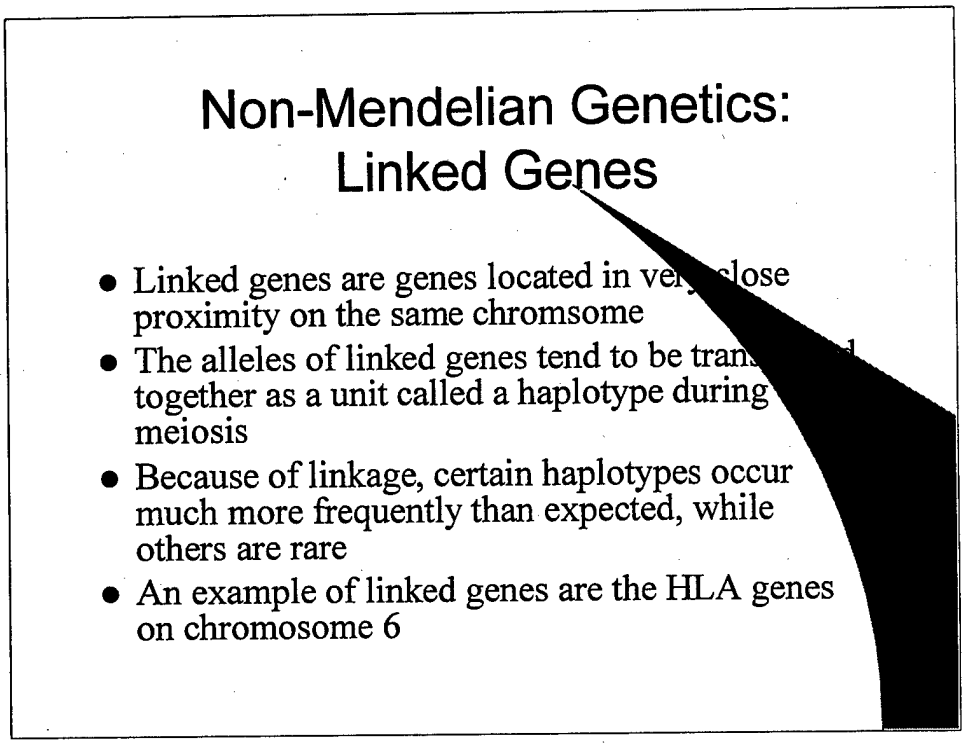
Genetic heterogeneity is when similar phenotypes are caused by different genotypes. Recognizing that this phenomenon can occur is an important aspect of clinical diagnosis in the field of medical genetics. An example is the inheritance of deafness. More than 132 forms of inherited deafness are known, yet two deaf people who are deaf because of a recessive disease can have normal hearing children if their deafness is caused by two different genes. Therefore, they have no chance of having deaf children instead of the normal 25% of offspring that would usually result from recessive inheritance.



In addition to the genes found along chromosomes in the nucleus, mitochondria also contain circular chromosomes with a small number of genes. Each egg cell of a female has many mitochondria in its cytoplasm, whereas each sperm has only several mitochondria. For this reason, a child inherits virtually all of his or her mitochondrial DNA from the mother. This also means that disease-causing mutations in mitochondria are only transmitted maternally. An example is Leber's hereditary optic neuropathy, which leads to blindness in young adulthood.



It is necessary not only to receive a pair of each chromosome, but also that one chromosome in a pair comes from a female and one from a male. This is because different genes are turned on/off depending on whether the chromosome the gene is on comes from a male or female. This process of sex-specific gene "silencing" is called imprinting. For imprinted genes, only the allele from either the mother or the father is expressed. If an offspring receives both copies of a certain portion of the genome from the same parent or if that part of the genome is deleted from one parent, the genes in that region will not function properly. Common diseases such as diabetes and psoriasis, as well as less common inherited diseases such as Prader-Willi and Angelman syndrome, call follow this unique inheritance pattern.



Usually genes independently assort due to being on different chromosomes or far apart on the same chromosome so that crossing-over can occur. However, when two genes are so close together on a chromosome that they do not independently sort because crossing-over cannot occur between them. These genes are said to be linked. The alleles of linked genes are typically inherited together as a unit, which alters the genetic ratios of these alleles. A combination of alleles from linked genes that tend to be inherited as a unit is referred to as a haplotype. Linkage explains why some haplotypes are more commonly observed in the population than others. The HLA gene cluster mentioned previously exemplifies these principles, which are exceptions to the typical Mendelian patterns of inheritance. Specific HLA alleles are associated with an increased risk for type 1 diabetes. Genetic risk for diabetes will be discussed in detail in Module 2.

Summary Questions

Greg and Marcia are both deaf due to inheriting a recessive condition, however all of their 7 children have normal hearing. Which answer below gives the best explanation for this phenomenon?

A) Codominance
B) Lethal alleles
C) Genetic heterogeneity
D) Linked genes

Leber's Hereditary Optic Neuropathy (LHON) is a mitochondrial disorder. Which of the following statements would be true about LHON t present in a family?

A) Only the females would have LHON
B) Only the males would have LHON
C) The females with LHON will have 100% affected childre some degree of LHON
D) The males with LHON will have 100% affected children w some degree of LHON

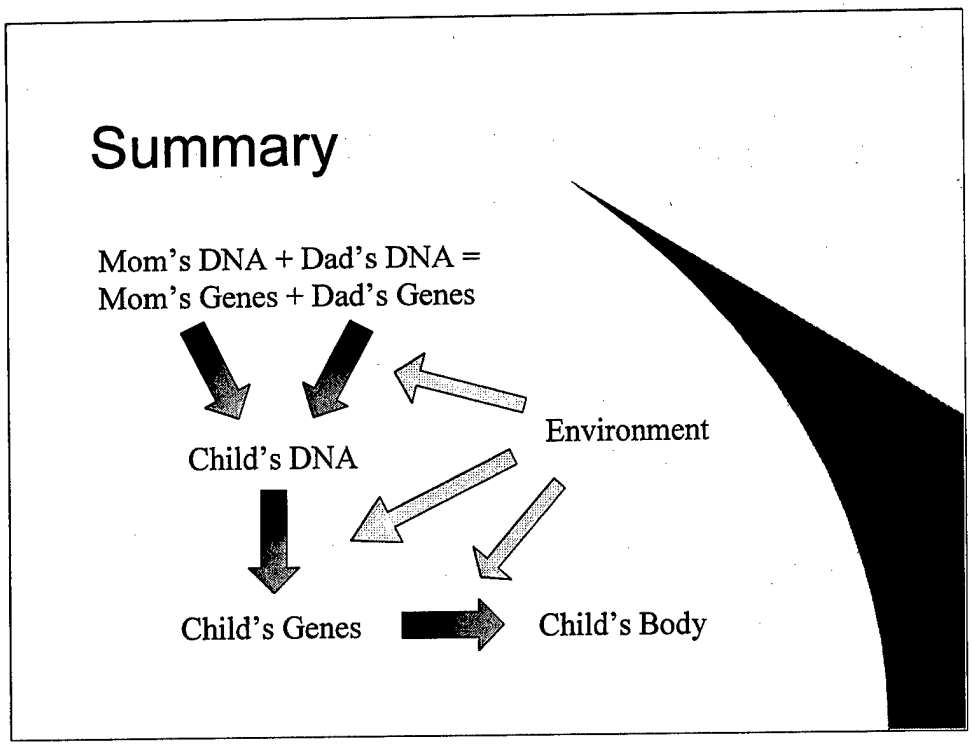
Answers

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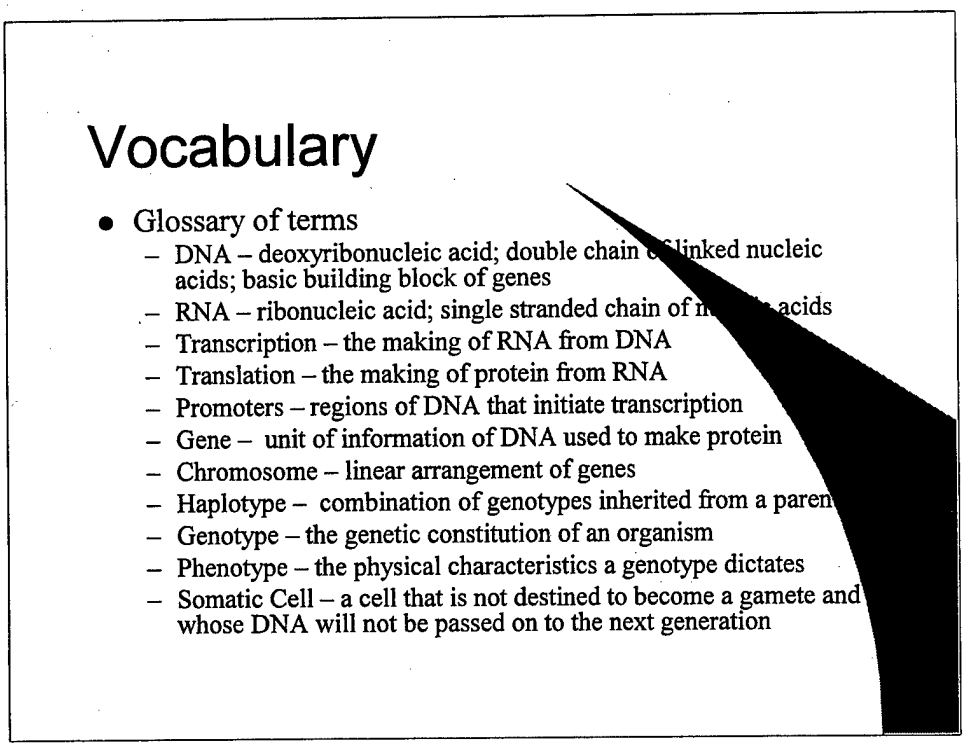
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A good way to think about all this is summed up in this diagram. The mother's DNA and the father's DNA make up their genes. When egg and sperm unite the mother's genes and the father's genes make their child's DNA which makes up their child's genes. The child's genes work to make the child's cells. The child's cells are what makes the child's body. Environmental factors can play a role at every step to alter the outcome.

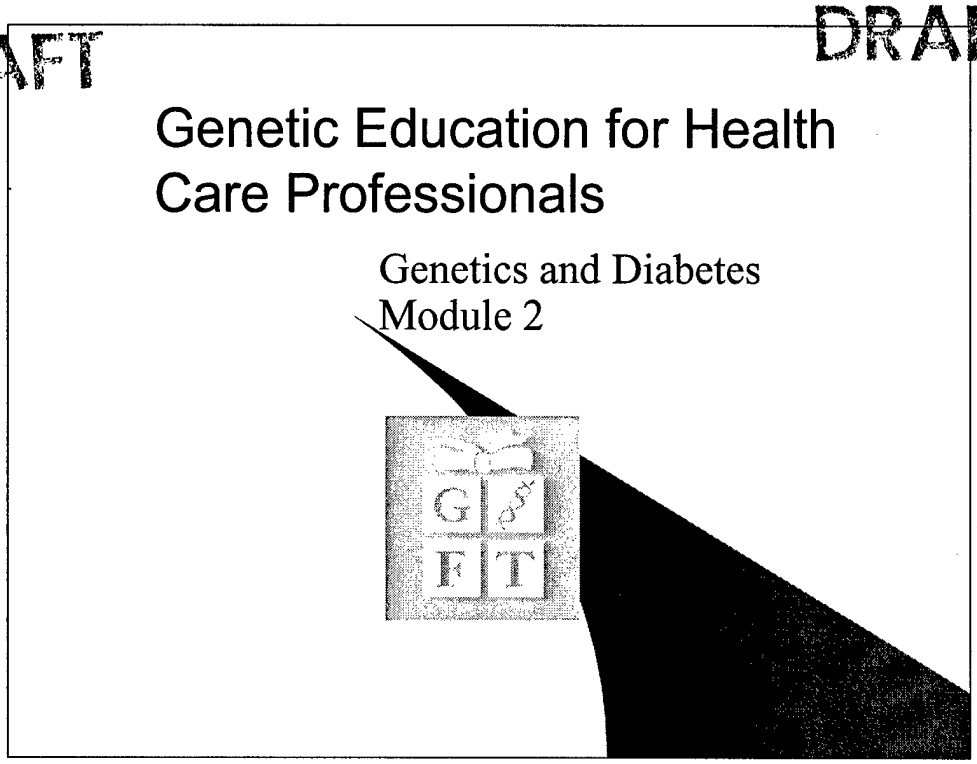


Perhaps this can be like GeneReviews where the word or term in the text is a hyperlink to the definition.

Vocabulary

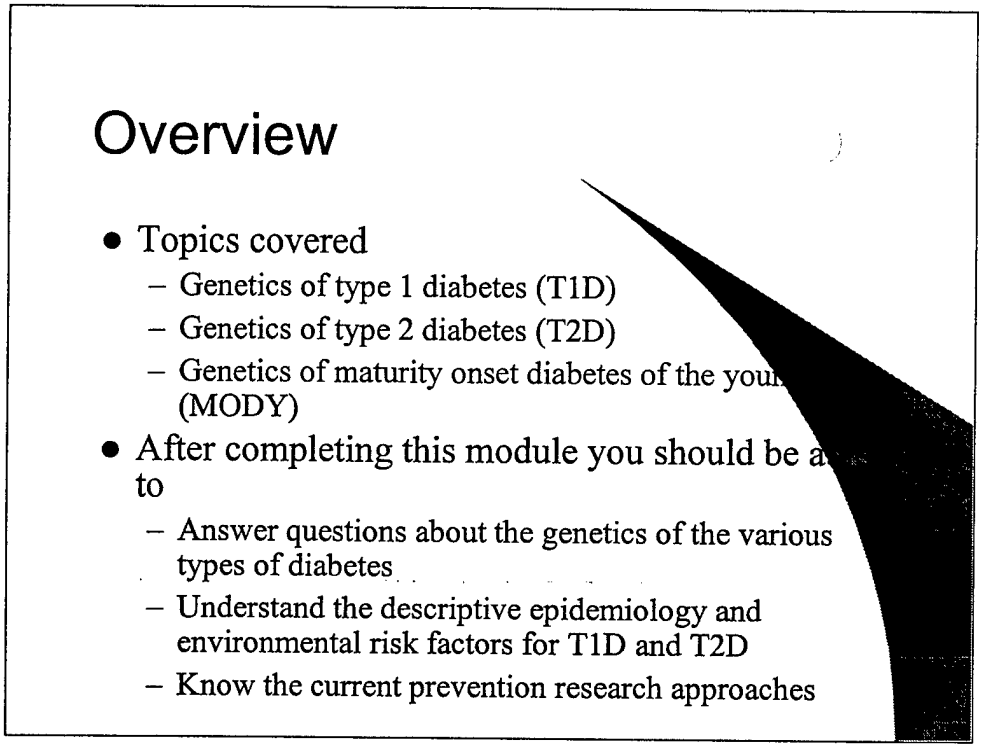
- Glossary of terms
  - Germ line – cell lineage in from which the gametes derive
  - Diploid – a cell with two chromosome sets
  - Haploid – a cell with one chromosome set
  - Mutation – change in genetic material
  - Point Mutations – change of a single base
  - Wild type – most common version of the DNA
  - Variant/mutant – non-wild type version of DNA
  - Deleterious mutation – decreases chances of survival
  - Beneficial mutation – enhances chances of survival
  - Conditional mutation – only affects phenotype under a defined set of conditions

DRAFT DRAFT



There are limited genetic educational opportunities for health care providers even though considerable information is available in the scientific literature, in books, and on the internet. It is becoming increasingly important for health care professionals to be knowledgeable about genetics because genetic testing is now receiving greater public attention, particularly through the media.

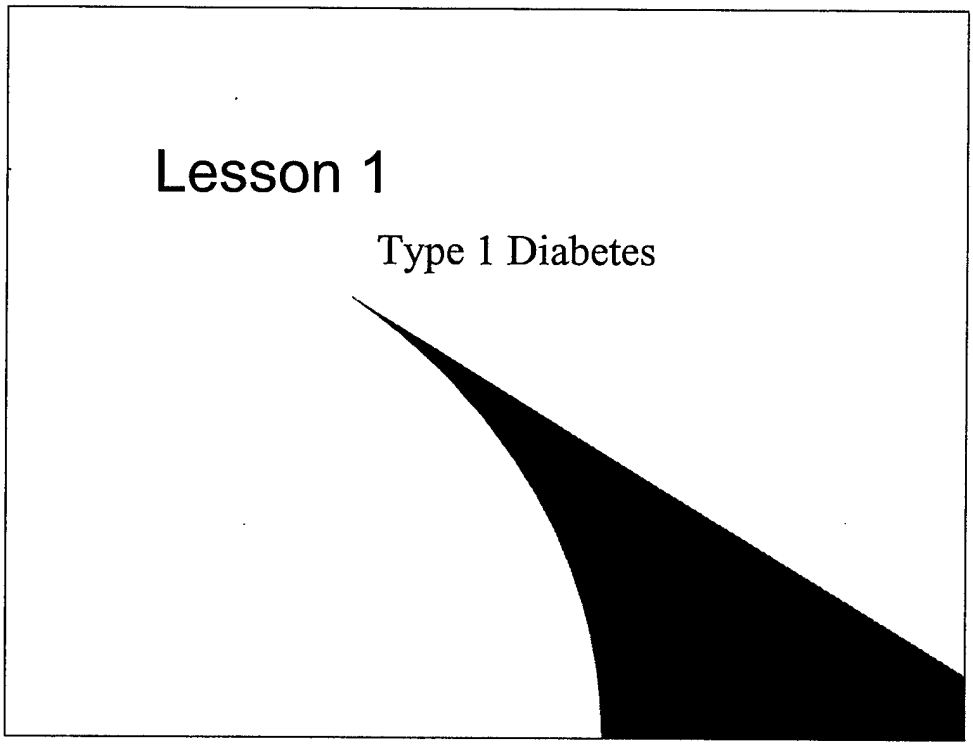
This module (Module 2) focuses on the genetics of diabetes. Individuals who do not have an understanding of basic genetics should review Module 1 prior to viewing Module 2.



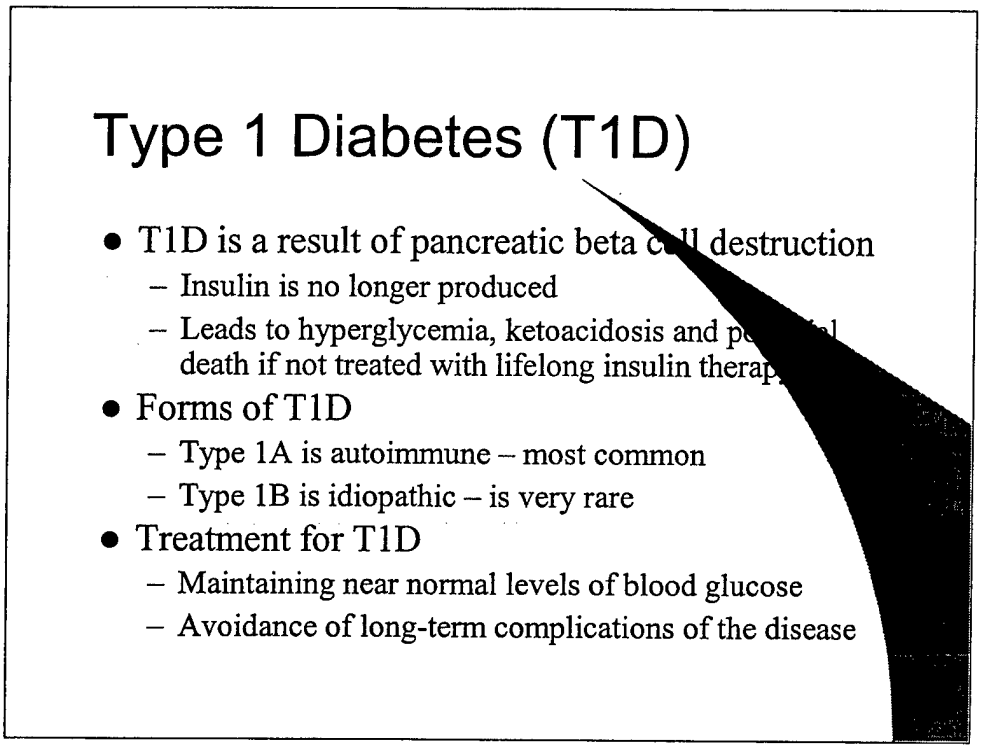
There are two major forms of diabetes: 1) type 1 diabetes (T1D), which occurs in approximately 10% of individuals who develop diabetes, and 2) type 2 diabetes (T2D), which is the form of diabetes that most people develop. The genetics of T1D and T2D are very different. There are also some rare forms of diabetes, such as maturity onset diabetes of the young (MODY) that can be directly inherited.

Because diabetes is a heterogeneous disease, it has been difficult to determine which genes are associated with the various types of the disease. In the future, identifying individuals who are at an increased genetic risk may permit the development of personalized intervention strategies that may have a higher likelihood of success than those targeted toward the general population. This includes determining which medications (based on knowing an individual's genetic profile) may be most appropriate for maintaining glycemic control.

After completing this education module, you will have an understanding of the genetic factors that contribute to increased susceptibility for the various forms of diabetes. You will also learn about the descriptive epidemiology and the environmental risk factors for T1D and T2D. Learning this information will increase your knowledge of the genetics of diabetes, and enable you to answer patients' questions about the genetics of diabetes.



The first section of Module I will focus on type 1 diabetes (T1D).

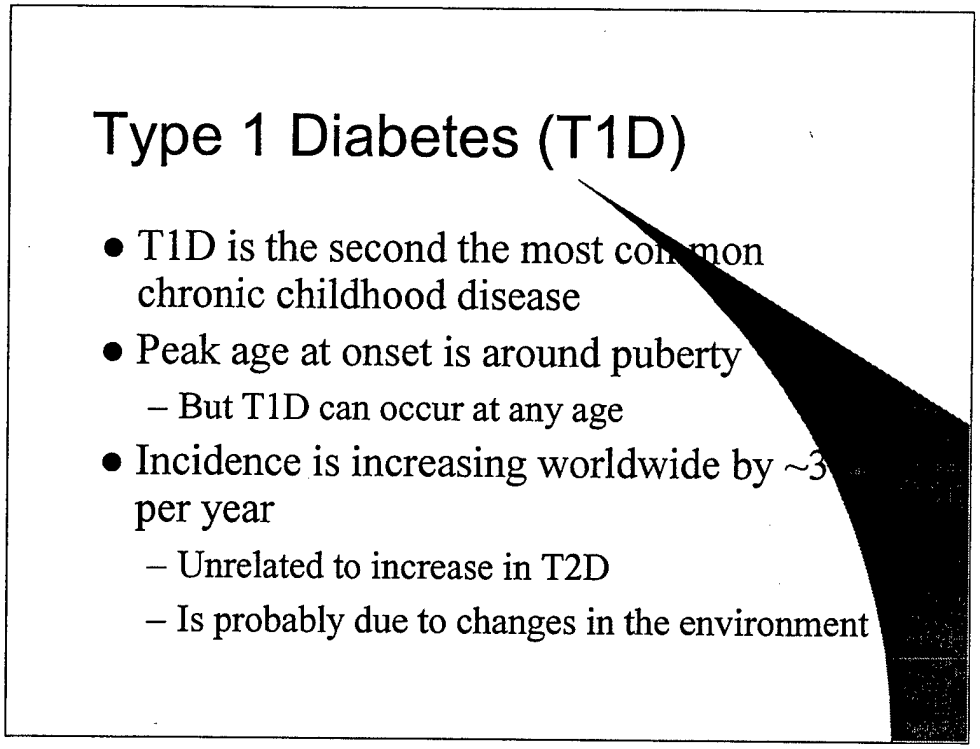


T1D develops as a result of the destruction of the beta-cells of the pancreas. It is characterized by absolute insulin deficiency, and may be fatal if not treated with lifelong insulin therapy. Severe complications at onset of T1D include hyperglycemia, ketoacidosis and even death.

In the past, T1D has also been called juvenile onset diabetes (JOD) because it is seen most often in children and young adults. It was also referred to as insulin-dependent diabetes mellitus (IDDM) because of the absolute requirement for life long insulin therapy. Today, it is called type 1 diabetes (T1D).

At the present time, two forms of T1D are recognized. Type 1 A is the more common form of the disease. It is immune mediated and characterized by the presence of autoantibodies to beta cell proteins, known as antigens. Type 1 B is very rare and is considered to be idiopathic because it may be a secondary complication of conditions like cystic fibrosis, or it may be induced by particular environmental toxins. When the term type 1 diabetes (T1D) is used in this Module, we are referring to type 1A.

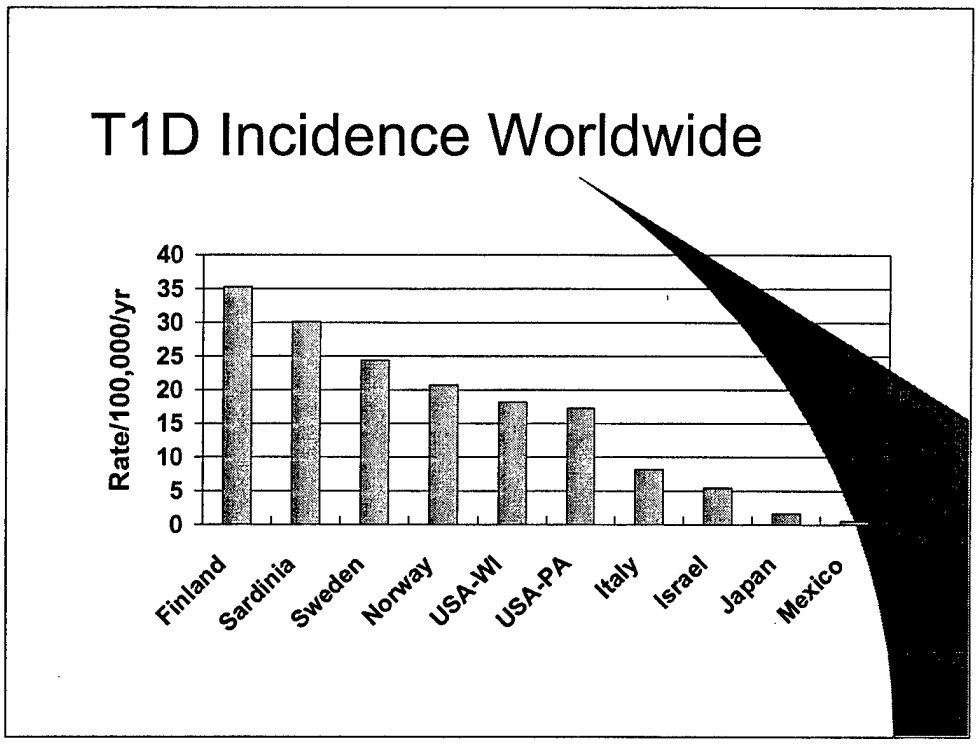
Treatment for T1D requires a strict regimen that is designed to maintain near normal levels of blood glucose. This typically includes a carefully calculated diet, planned physical activity, home blood glucose testing several times a day, and multiple daily insulin injections. Individuals with good glycemic control are less likely to develop some of the serious long-term complications of the disease which include cardiovascular peripheral vascular ocular neurologic



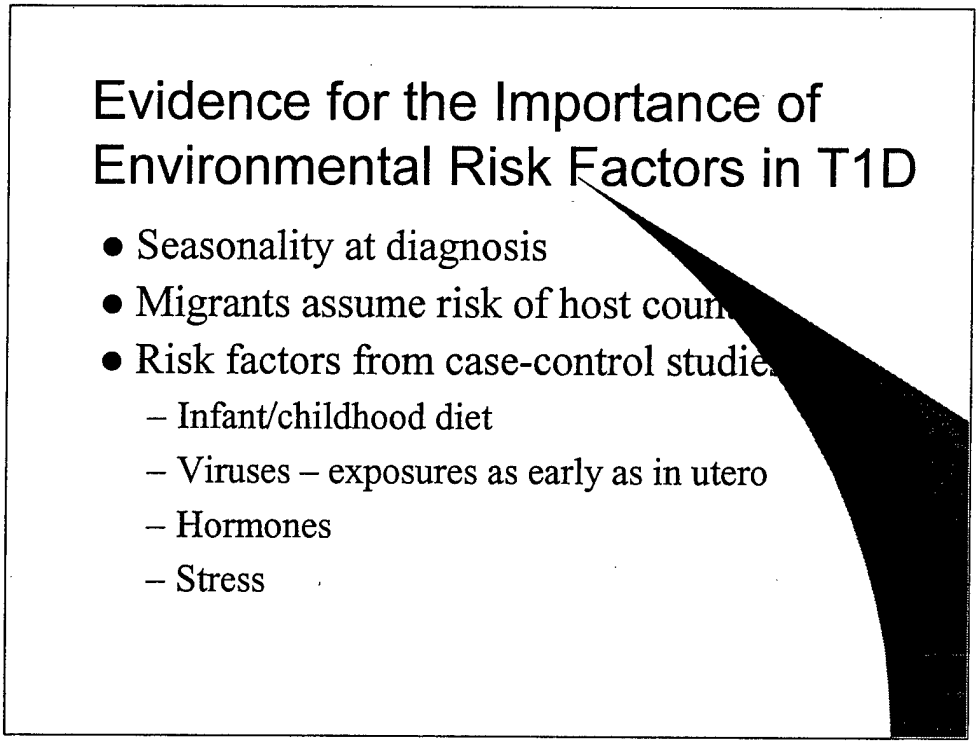
T1D is the second most common chronic childhood disease. The only disease with a higher prevalence in children is asthma. It has been estimated that approximately 20 million people worldwide have T1D

The peak age at onset of T1D is around the time of puberty, and generally occurs earlier in girls than in boys. Although T1D is generally diagnosed in children and young adults, it can occur at any age. More is known about the epidemiology of diabetes in children compared to adults because it has been easier to identify and study affected children (e.g., ascertained through pediatricians) than affected adults, who are treated by a variety of medical specialists, including primary care physicians, endocrinologists, out patient clinics, etc.

The incidence of T1D is increasing around the world at a rate of about 3% per year, reasons for which are currently unknown. This trend appears to be most dramatic in the youngest age groups, and is completely unrelated to the current increase in T2D in children. Researchers have speculated about possible environmental changes (e.g., air pollution, improved hygiene, infant feeding patterns) that may explain the rising incidence. These increasing T1D incidence is unlikely to be due to genetic changes, because it takes many generations to alter population gene frequencies. Additional research is needed to determine why the incidence of autoimmune T1D is increasing worldwide.



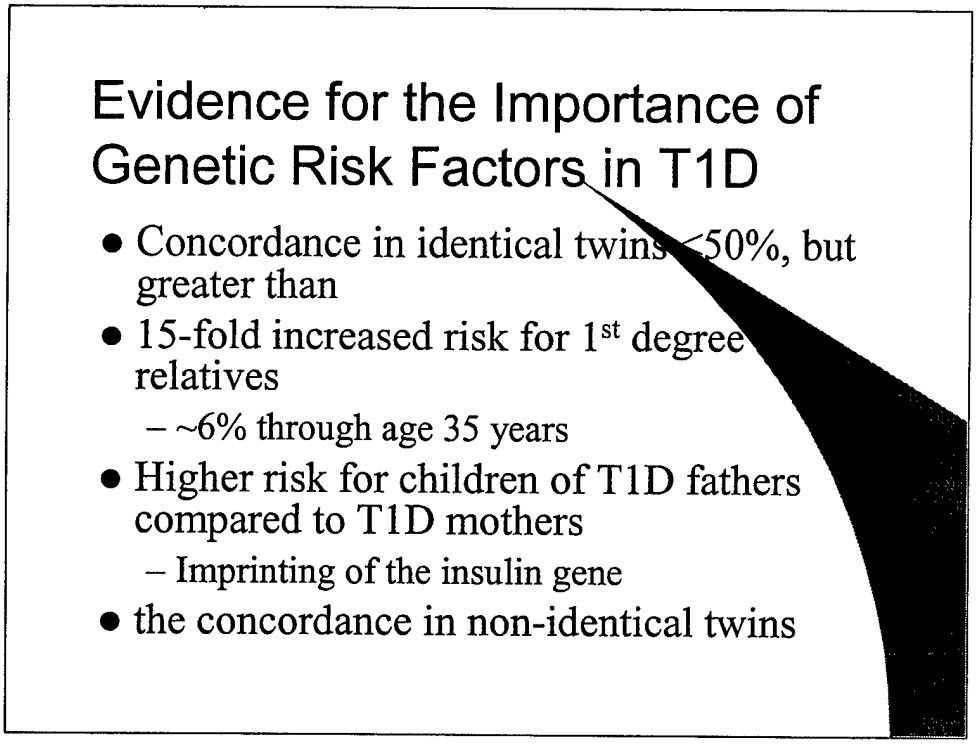
This figure characterizes the dramatic geographic differences in T1D incidence. Countries with the highest incidence include Finland, Sardinia, and Scandinavia. Those with lowest are found in Asia. However, Native-Americans and some populations in Latin America have an extremely low incidence of the disease. The dramatic difference in T1D incidence worldwide are likely related to variations in the prevalence of specific genetic and environmental risk factors for the disease.



In addition to the evidence already provided, there are other epidemiologic patterns that suggest that environmental factors are important in the etiology of T1D. The diagnosis of T1D is more common during the cold winter month compared to the summer. This parallels the seasonal patterns observed for infectious diseases, which have been suggested as risk factors.

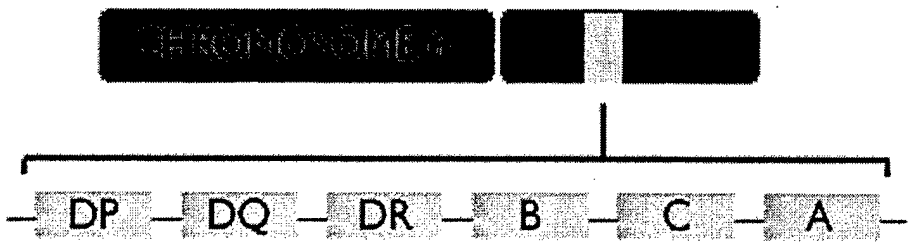
In addition, when children from countries with a low T1D incidence rate migrate to countries with a high T1D incidence rate, their risk increases and becomes similar to that for the host country. This difference is much less dramatic for individuals who migrate during their adult years, indicating that the childhood exposures are probably most diabetogenic.

Studies that have compared children with T1D to unrelated non-diabetic children (i.e., case-control studies) have shown that several environmental risk factors are important in the etiology of T1D. These include infant / childhood diet. For example, breast feeding appears to be protective, and early exposure to cow's milk increases T1D risk. A number of viral infections have also been associated with T1D. These include those that occur *in utero*, as well as those that typically occur during childhood. Because the peak onset of T1D is at puberty, it is thought that changing levels of hormones may also precipitate the disease. Finally, stress has been suggested as a T1D risk factor. Many parents report that their child experienced a very stressful event (e.g., divorce) just prior to the onset of the disease.



There is also evidence that T1D is, in part, a genetic disorder. Identical twins (i.e., monozygous twins) are more likely to both have T1D than non-identical twins (i.e., dizygous twins). But concordance rates in identical twins are less than 50%, supporting the hypothesis that environmental factors are also important in the development of T1D.

Siblings of an individual with T1D are about 15 times more likely to develop T1D than individuals in the general population. This translates to a cumulative risk of approximately 6% through age 35 years. Interestingly, children in families with a T1D mother have a lower risk (~3%) than children in families with a T1D father, reasons for which are unknown. One theory is that imprinting plays a role in this phenomenon. Imprinting refers to the lack of expression of a gene depending on the gender of the parent who transmits it. When a gene is expressed, it is used to make protein. If a maternal gene is imprinted, it is turned off and the paternal gene will be expressed. The converse is true if the paternal gene is imprinted; the gene from the father is turned off and the gene from the mother is expressed. For example, it is known that the insulin (*INS*) gene is located in a region that is maternally imprinted (chromosome 11p15.5, where maternal genes are turned off), but the paternal *INS* gene is expressed. This may explain the increased risk for children of diabetic fathers.



CELL SURFACE PROTEINS:

CLASS I HLA-A, HLA-B, HLA-C

CLASS II HLA-DR, HLA-DQ, HLA-DP

Figure 4. HLA is a major biological barrier to transplantation. The HLA genes are found on chromosome 6.

Particular human leukocyte antigen (HLA) genes also influence an individuals risk of developing T1D. These genes are located on the short arm of chromosome 6. There are 2 major types of HLA genes – class I and class II. HLA class I genes (HLA A, C and B) code for molecules that are expressed on all nucleated cells and are important in determining immune response. HLA class II genes code for molecules that are expressed on the surface of B lymphocytes, macrophages, and other antigen-presenting cells that also control immune response. There are 3 major class II loci; HLA-DR, HLA-DQ, and HLA- DP. It is unclear whether: 1) HLA class I genes increase T1D risk independently of class II genes, or 2) their association is due to the strong linkage disequilibrium (the occurrence of genes together on the same chromosome more often than would be expected) that occurs between these HLA regions.

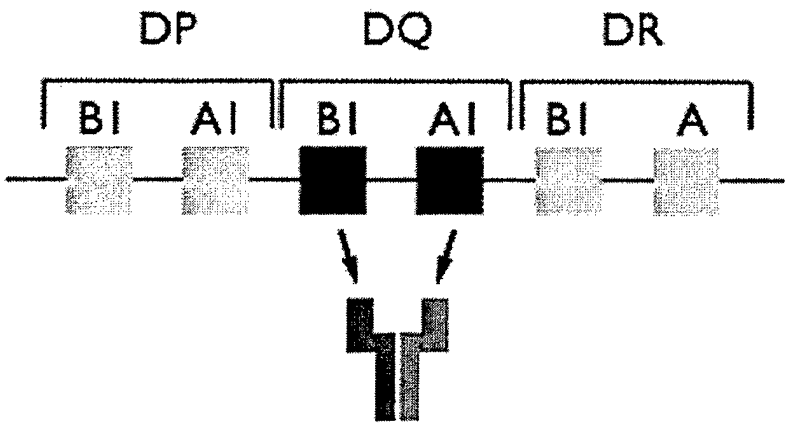


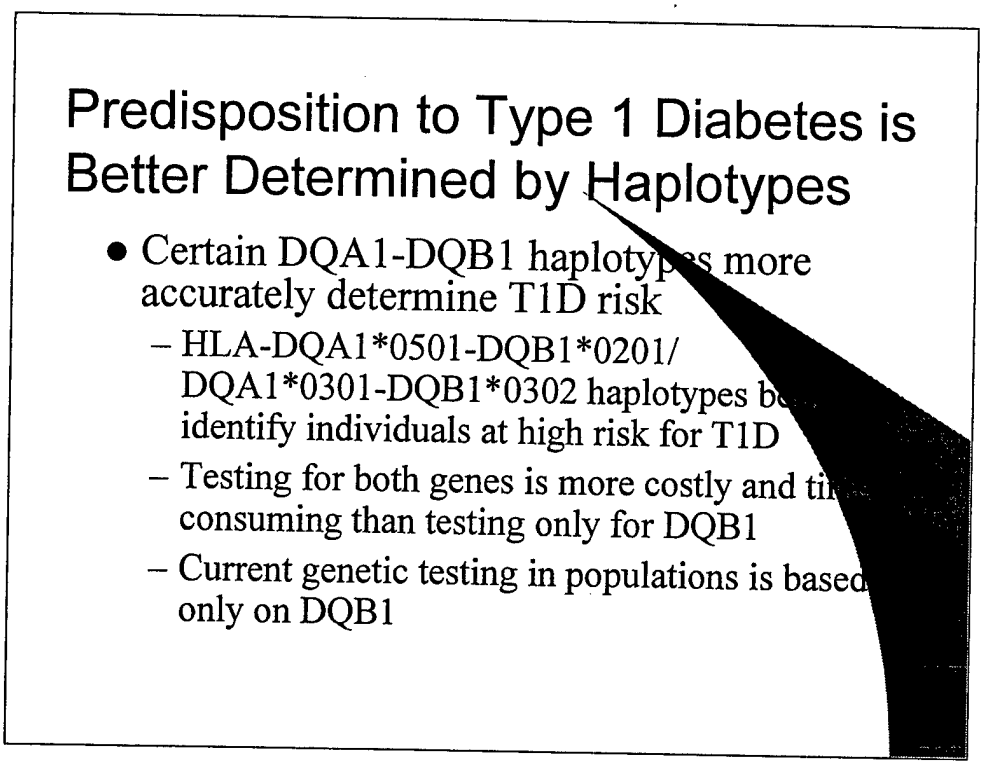
Figure 5. The products specified by the DQA1 and the DQB1 genes form the DQ antigen.

The best single genetic markers for T1D are located at the HLA-DQ locus, particularly the HLA-DQB1 gene.

The DQB1 gene codes for only half of the DQ molecule (i.e., the DQ beta chain). The other half of the DQ molecule (i.e., the DQ alpha chain) is encoded by the DQA1 gene. Both genes are very polymorphic. The DQA1 locus also affects T1D risk; but it's effect is less dramatic than that of DQB1.

The alleles associated with increased T1D risk are DQB1*0201 and DQB1*0302. About 20% of Caucasians in the general population carry DQB1*0201. The corresponding frequency for DQB1*0302 is around 10%. However, the frequency of these alleles varies across ethnic groups. In contrast to what is observed for the general population, about 90% of Caucasians with T1D carry at least one of these high risk alleles.

The DQB1*0602 allele is rarely found in individuals with T1D, but is present in ~13% of the general Caucasian population. This allele actually decreases risk of developing the disease.



Because DQB1 is the best single genetic marker for T1D risk it is the gene most often used to identify individuals with a high risk of developing disease. However, risk estimates based on DQB1 alone are less precise than those based on the combination of alleles at both the DQA1 and DQB1 loci. These combinations are called haplotypes. Not all haplotypes with DQB1*0201 or *0302 also have high risk DQA1 alleles. Thus, DQB1 and DQA1 typing provide more accurate risk estimates than those based on DQB1 alone. However, it is also more expensive.

The two DQA1-DQB1 haplotypes that are most strongly associated with T1D are DQA1*0501-DQB1*0201 and DQA1*0301-DQB1*0302. That is, chromosomes with DQB1*0201 and DQA1*0501 confer a higher risk for T1D than chromosomes with DQB1*0201 but some other DQA1 allele (not *0501). Similarly, chromosomes with DQB1*0302 and DQA1*0301 confer a higher risk for T1D than chromosomes with DQB1*0302 and another DQA1 allele (not *0301).

Relative Increase in T1D Risk for Individuals with High Risk DQA1-DQB1 Haplotypes

	Relative Increase in Risk	
	Two high risk haplotypes	One high risk haplotype
Caucasians	16	4
African Americans	45	7
Asians	11	4

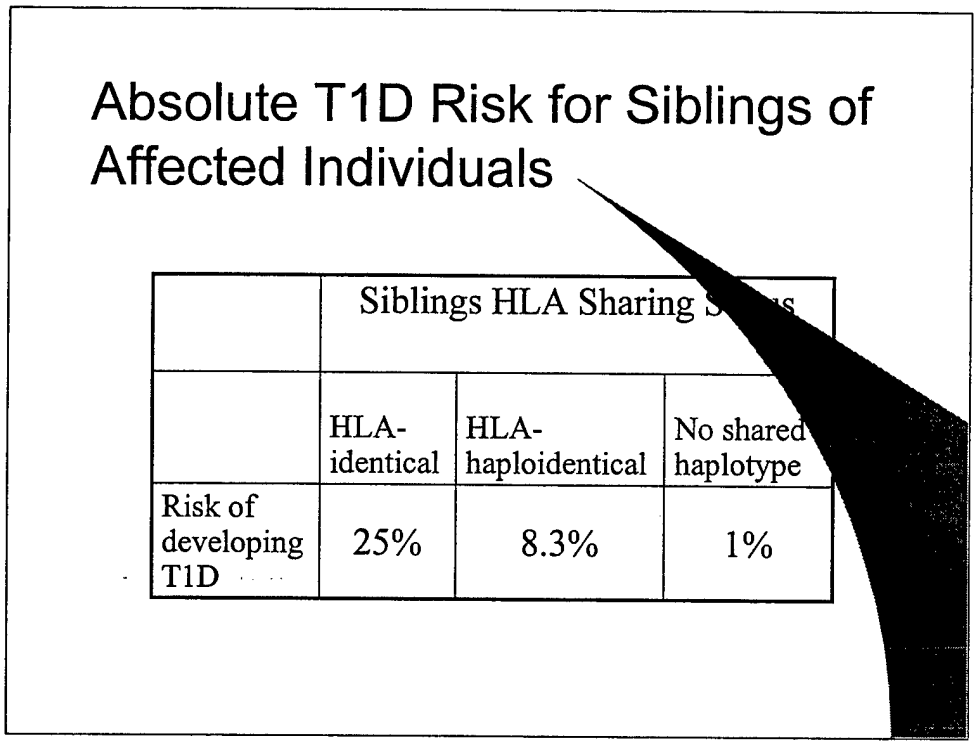
This table illustrates the relative increase in risk depending on the number of high risk DQA1-DQB1 haplotypes an individual carries. For example, Caucasians with two high risk haplotypes have a 16-fold higher T1D risk than an individual who has no high risk haplotypes. The risk for Caucasian individuals with one high risk haplotype is about 4 times higher that the risk for someone with no high risk haplotypes. This also means that Caucasians with two high risk haplotypes are 4 times more likely to develop T1D than those with one high risk haplotype; and individuals with one high risk haplotype are about 4 times more likely to develop T1D than those with no high risk haplotypes.

As illustrated, the magnitude of the relative increase in risk differs by ethnicity. However, the same trends are apparent – those with two high risk haplotypes are most likely to develop T1D.

Absolute T1D Risk through Age 30 Years

	Number of High Risk DQA1-DQB1 Haplotypes		
	Two	One	
Caucasians	2.6%	0.7%	0.2
African Americans	3.1%	0.5%	0.1%
Asians	0.2%	0.1%	0.02%

This table illustrates the absolute risk of developing T1D depending on the number of high risk haplotypes an individual carries. For example, Caucasians and African Americans with two high risk haplotypes have about a 3% chance of developing the disease before age 30 years, depending on the population. The risk for Asians with two high risk haplotype is much lower (less than 1 %). Therefore, even if a person carries high risk haplotypes, their chances of developing T1D are quite low.



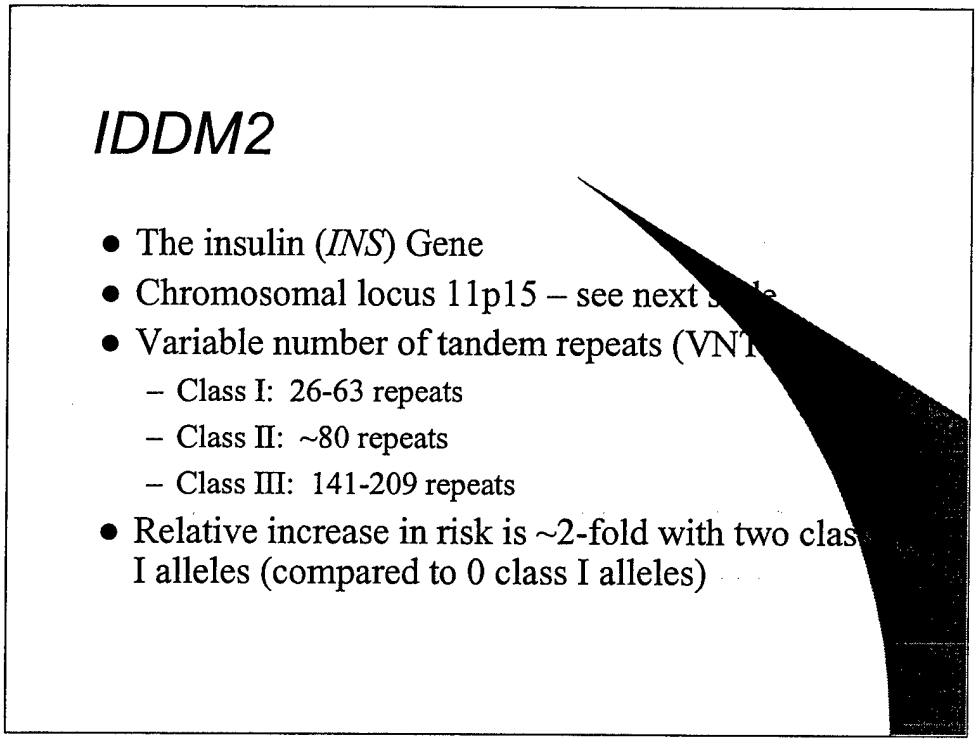
In families where there is already one person with T1D, the risk to unaffected relatives is higher than in families where no one has T1D. Instead of determining how many high risk haplotypes an individual carries, one often looks at whether the haplotypes carried by unaffected siblings are the same as those carried by the affected relative. An unaffected sibling who shares both haplotypes with the person with T1D is referred to as 'HLA-identical'. If they only share one haplotype, they are know as 'HLA-haploidentical'.

As can be seen, the T1D risk for someone who is HLA-identical is quite high (about 25%). The risk is about 8% for siblings who share only one haplotype. The fact that the risk for individuals who have no shared HLA haplotype (about 1%) is still increased compared to the prevalence in the general population suggests that genes other than those in the HLA region also increase susceptibility for the disease.

Genome Screens for T1D

IDDM1	6p21	*IDDM13*	2q34-q35
IDDM2	11p15	*IDDM15*	6q21
IDDM3	15q26	*IDDM17*	[illegible]25
IDDM4	11q13	*IDDM18*	5q[illegible]
IDDM5	6q25-q27		1q42-[illegible]
IDDM6	18q21		8q24
IDDM7	2q31	VDR, INFγ	12q12-qte[illegible]
IDDM8	6q27-qter		16p11-p13[illegible]
IDDM9	3q21-q25		16q22-q24
IDDM10	10p11-q11		17q24-qter
IDDM11	14q24-q31	TGFβ1	19p13-q13
IDDM12	2q33		Xp11

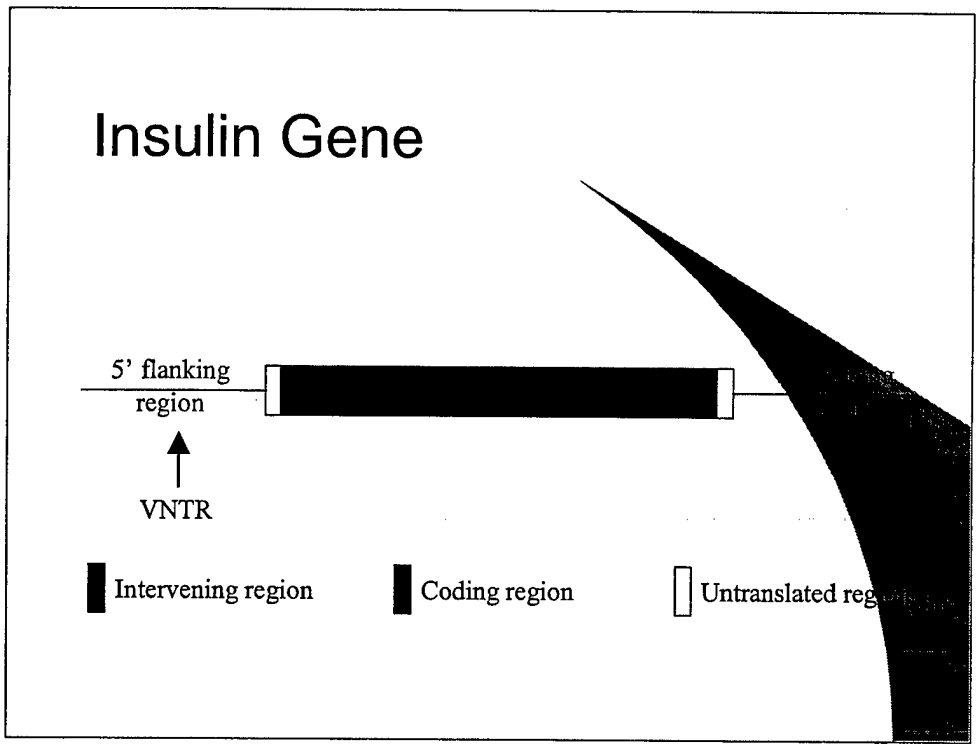
A number of studies have screened the entire human genome in families with more than one T1D individual to identify other regions that play a role in determining one's level of genetic susceptibility for T1D. The loci listed in this table represent a summary of those that are most likely to contain candidate genes. However, none of these loci have effects that are as strong as the HLA genes in determining T1D risk. As a result, the HLA regions has been named *IDDM1*.



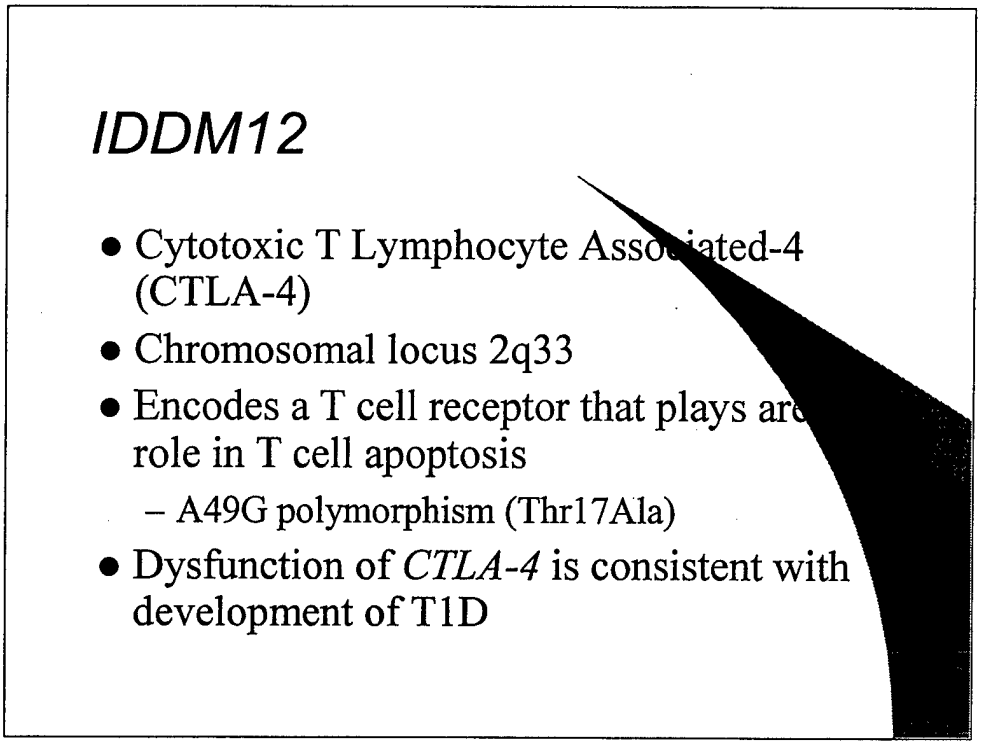
The next strongest locus involved in genetic susceptibility to T1D is *IDDM2* on the short arm of chromosome 11. This locus encodes the insulin gene (*INS*). The 5' regulatory region of the insulin gene region contains a variable number of tandem repeat (VNTR) locus that has been associated with T1D. There are 3 classes of VNTR alleles: class I (26-63 repeats), class II (~80 repeats), and class III (141-209 repeats). The next slide is a cartoon of the VNTR.

A person who carries two class I alleles has about a two-fold increase risk of developing T1D (compared to those who carry no class I VNTRs). Class III alleles seem to provide dominant protection against developing T1D. Class II alleles are virtually non-existent in Caucasian populations and have no effect on T1D risk.

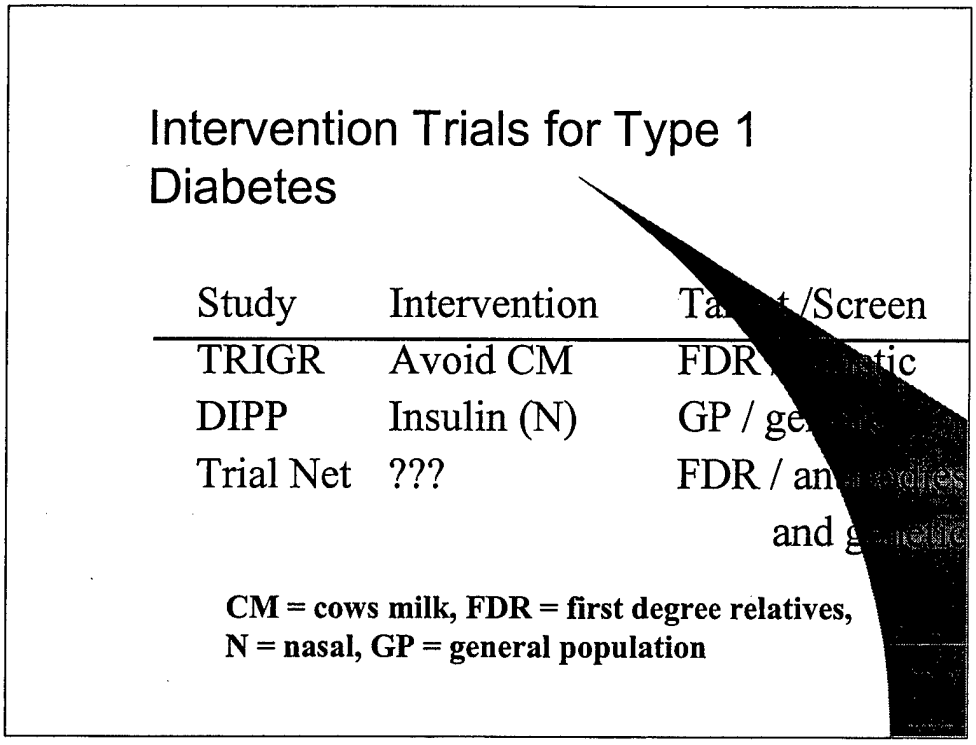
The insulin locus and the other candidate regions identified by genome screens require further evaluation studies before they can be included in genetic tests for T1D.



This figure depicts the structure of the *INS* gene. The variable number of tandem repeats region is located in the 5' flanking region.



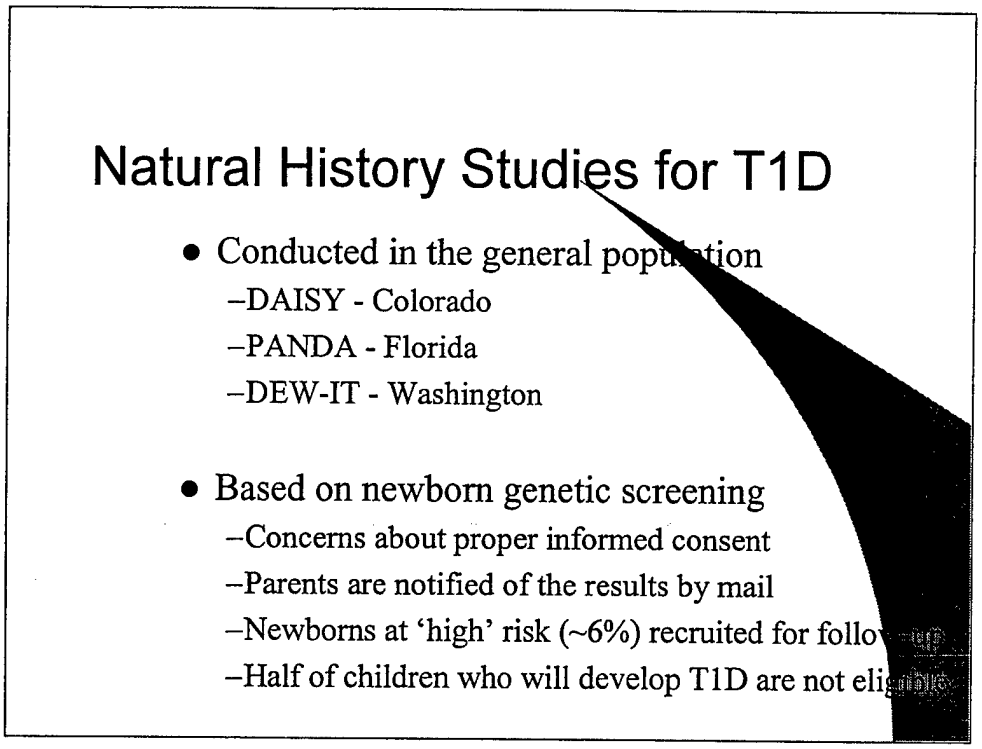
The *CTLA-4* gene region on chromosome 2q33 has also been associated with T1D. The locus that includes the *CTLA-4* gene has been termed *IDDM12* (see slide summarizing genome screens). *CTLA-4* is a strong candidate gene for autoimmune diseases because it encodes a T cell receptor that plays a role in controlling T cell apoptosis (programmed cell death) and is a negative regulator of T cell activation. Specifically, an A49G (adenine/guanine) polymorphism within the first exon of the *CTLA-4* gene was found to be associated with development T1D. This polymorphism was found to be important in Italian, Spanish, French, Mexican-American, and Korean populations.



Although a cure for T1D is currently unavailable, several large multi-national investigations have been designed to evaluate a number of primary and secondary disease interventions. Two are targeted towards unaffected first degree relatives (FDR) in families with an individual with T1D. One is targeted to the general population (GP) of Finland.

Newborns at high risk of developing T1D are identified by genetic testing (for high risk DQB1 alleles) for the Trial to Reduce Type 1 Diabetes in Genetically at-Risk (TRIGR) and the Diabetes Prediction and Prevention Project (DIPP). For TRIGR, newborns are randomized to receive either a regular cow's milk formula or one with only hydrolyzed proteins, which is thought to be protective. This study is being conducted in Europe and North America. For DIPP, newborns with high risk DQB1 alleles from the general population of Finland are followed until the become beta cell antibody positive. When this occurs, they are randomized to an intervention based on nasal insulin.

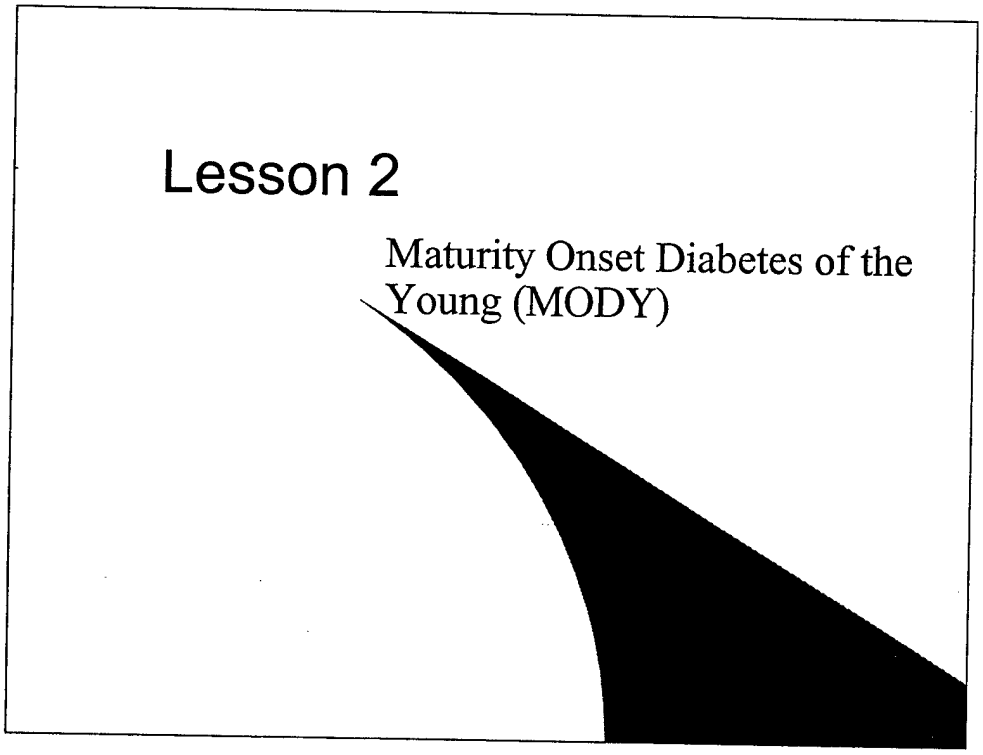
New interventions that will be tested in the future will be conducted through T!D Trial Net, a collaborative network of clinical centers and experts in diabetes and immunology. These studies will identify unaffected FDR with evidence of beta cell destruction and who do not carry protective the DQB1*0602 gene), who will be eligible for new interventions.



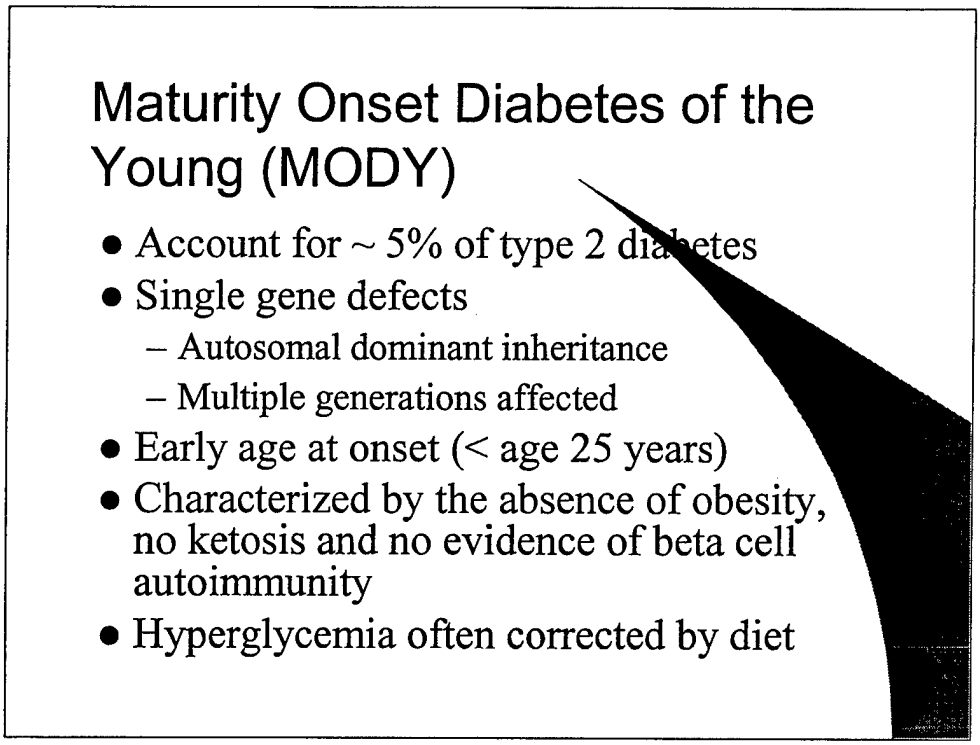
In addition to the intervention trials, there are also three natural history studies for T1D that are ongoing in the US. These include DAISY (Diabetes Autoimmunity in the Young Study) in Colorado, PANDA (Prospective Assessment in Newborn of Diabetes Autoimmunity) in Florida, and DEW-IT (the Diabetes Evaluation in Washington). All are based on newborn genetic screening, and therefore, concerns have been raised about proper informed consent.

Parents of babies who carry high risk DQB1 alleles receive a letter informing then that their infant is at 'high' or 'moderate' risk of developing the disease. The likelihood that these children will develop T1D before they are 35 years of age is actually only about 6%. Moreover, approximately half of the children who actually will develop T1D come from the 'low' risk group, which is not eligible to participate.

The final report of the Task Force on Genetic Testing recently noted that "'a knowledge base on genetics and genetic testing should be developed for the general public. Without a sound knowledge base, informed decision are impossible and claims of autonomy and informed consent are suspect". Because genetic testing for T1D is currently being conducted, it is essential that we begin to address these issues now. We developed this Module with the hope that it will begin to fulfill the need for health professionals who work with families with diabetes. Module 3 will cover informed consent, psychosocial and ethical issues that are related to diabetes and the actual process of genetic testing.



The second section of Module 2 will focus on Maturity Onset Diabetes of the Young (MODY).



Another type of diabetes that occurs in children and young adults is maturity onset diabetes of the young (MODY). MODY is a rare form of T2D, and accounts in only ~5% of all T2D cases. MODY is caused by a mutation in a gene involved in pancreatic development, insulin gene expression, or glucose sensing. MODY follows an autosomal dominant pattern of inheritance. Thus, an affected individual only carries one copy of the mutant gene, and in families, MODY it is frequently observed in every generation. Asymptomatic carriers in MODY families will almost certainly develop the disease at a later age. MODY is characterized by the absence of obesity, no ketosis and no evidence of beta cell autoimmunity.

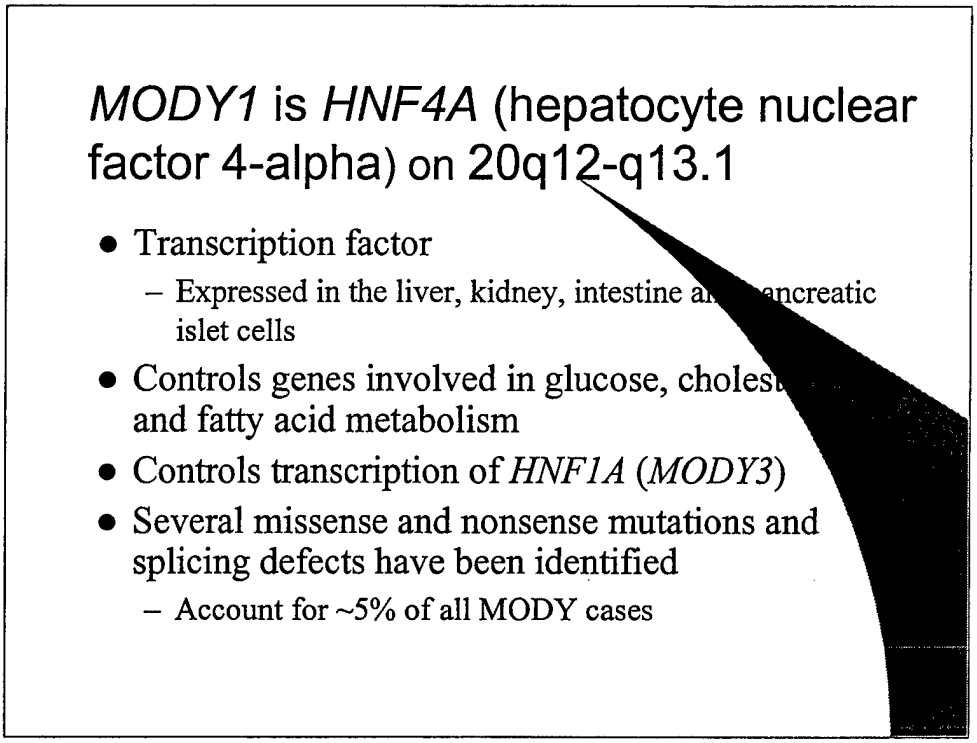
MODY generally presents as mild type 2 diabetes and is most often diagnosed before age 25 years. Because MODY occurs in children, it is sometimes misdiagnosed as T1D. Young women with MODY are also frequently diagnosed during pregnancy. MODY often can be managed without insulin although some individuals with MODY will need to have insulin injections at a later age.

Maturity Onset Diabetes of the Young (MODY) Genes

Type	Gene	Locus	Protein	# Mutations	% MODY
MODY1	*HNF4A*	20q12-q13.1	Hepatocyte nuclear factor 4-alpha	12	~5%
MODY2	*GCK*	7p15-p13	Glucokinase	~	~15%
MODY3	*HNF1A*	12q24.2	Hepatocyte nuclear factor 1-alpha	>100	
MODY4	*IPF1*	13q12.1	Insulin promotor factor-1	Few	
MODY5	*HNF1B*	17cen-q21.3	Hepatocyte nuclear factor 1-beta	Few	
MODY6	*NEUROD1*	2q32	Neurogenic differentiation factor 1	Few	

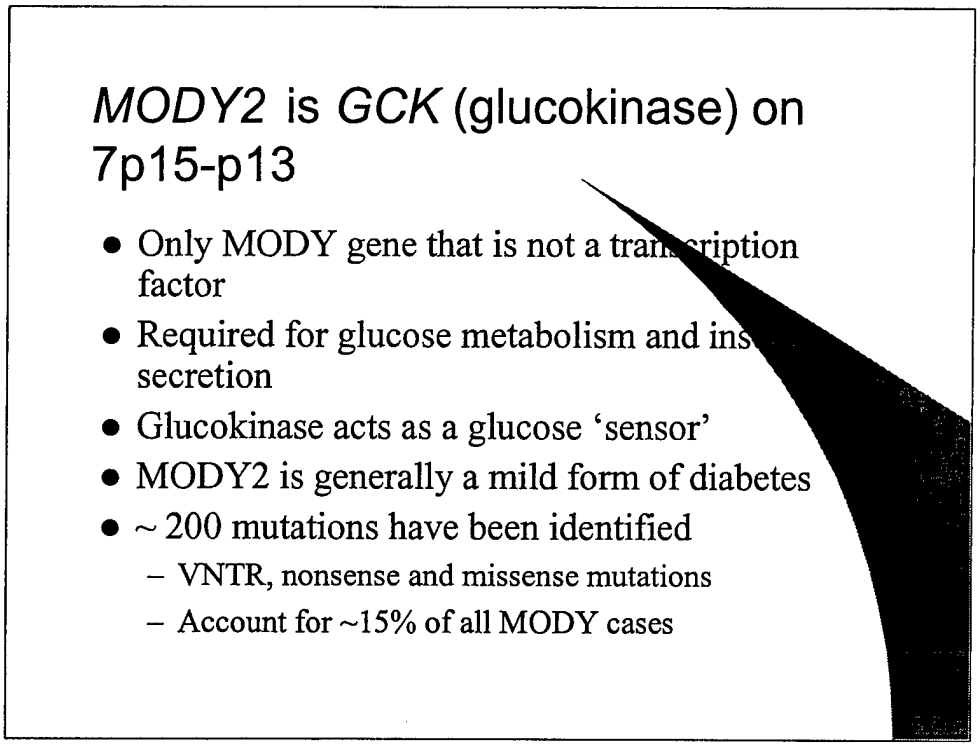
This table summarized the genes involved in the 6 different types of MODY, their chromosomal locus, the protein encoded by the gene, the number of mutations identified, and the proportion of MODY cases caused by mutations in the gene. It is known that additional MODY genes have yet to be identified, as about 15% MODY families do not carry one of the confirmed mutations.

Each known MODY gene will be described separately in a subsequent slide.



MODY1 is caused by mutations in the *HNF4A* gene (OMIM 600281), which encodes the hepatocyte nuclear factor 4-alpha gene on chromosome 20q12-q13.1. It is also known as TCF14 (transcription factor 14). *HNF4A* is a transcription factor that is expressed in the liver, kidney, intestine and pancreatic islet cells. It controls expression of genes involved in glucose, cholesterol and fatty acid metabolism, including glucose transporter 2, aldolase B, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase. It also regulates transcription of the transcription factor *HNF1A* (OMIM 142410), which is the gene that causes MODY3.

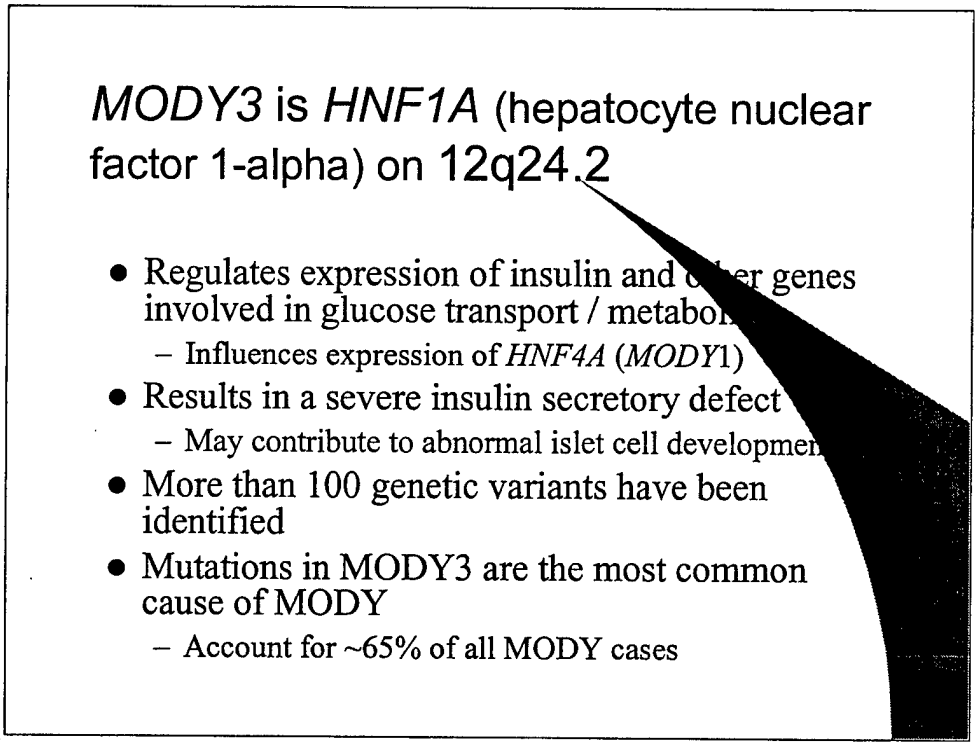
HNF4A may also play a role in NIDDM, particularly in individuals with a young age at onset. Approximately 12 missense and nonsense mutations, as well as a splicing defect, in *HNF4A* have been identified in families with *MODY1*. All appear to reduce gene expression. *MODY1* accounts for about 5% of all MODY cases.



MODY2 is due to mutations in the glucokinase gene (*GCK;* OMIM 138079) on chromosome 7p15-p13. This is the only MODY gene that does not regulate the expression of other genes. Rather, the GCK gene is required for glucose metabolism and insulin secretion. Thus, the clinical course of MODY2 patients differs from the prognosis associated with other types of MODY.

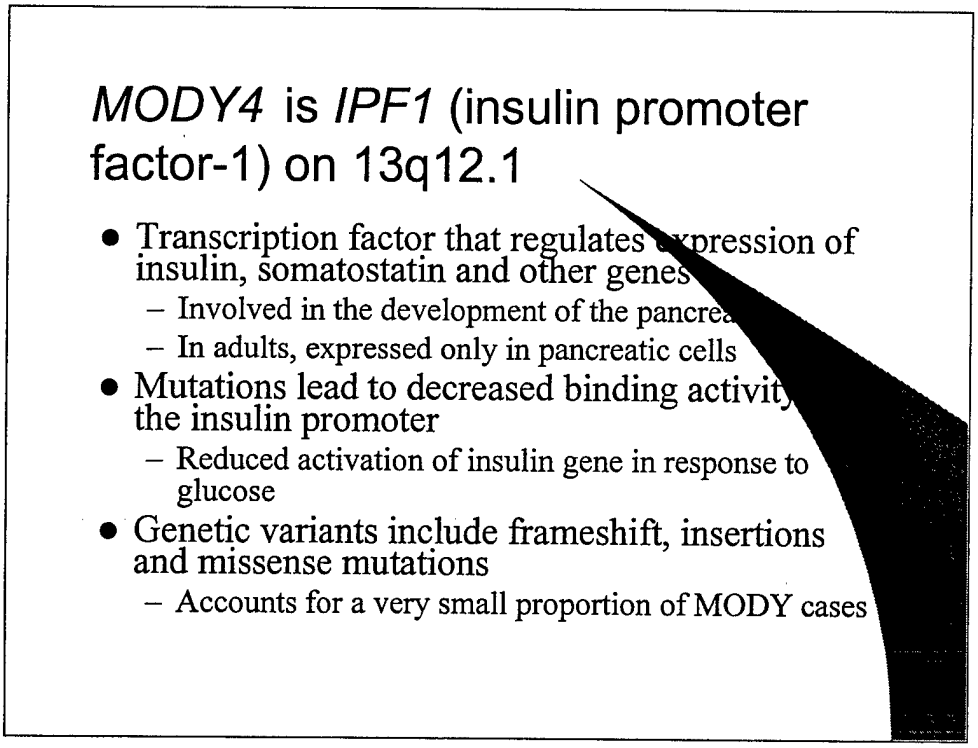
Glucokinase alerts pancreatic beta cells that glucose is present, and therefore acts as a glucose sensor. Mutations in this gene reduce the beta-cell secretory response to glucose, which leads to chronic hyperglycemia.

MODY2 is characterized by a mild stable fasting hyperglycemia with a decline in pancreatic islet cell function over time. It has also been associated with gestational diabetes, T2D, and possibly T1D. Many patients with *MODY2* are treated with diet alone. Approximately 200 genetic variants have been identified, including VNTR polymorphisms, nonsense and missense mutations. *MODY2* accounts for ~15% of all MODY cases.



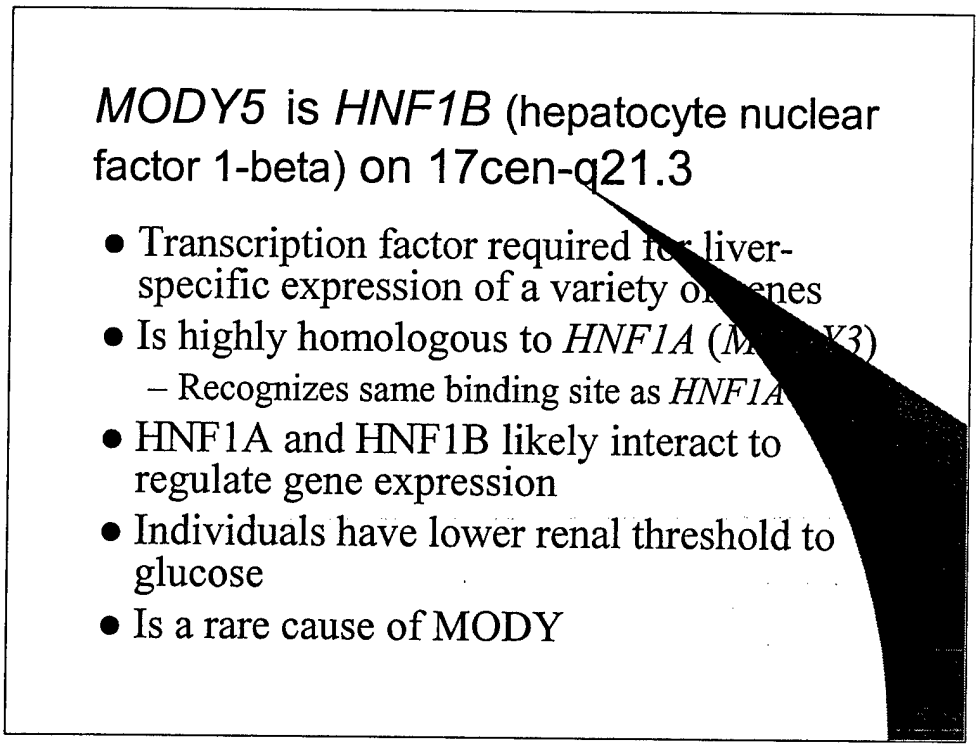
MODY3 is located on chromosome 12q24.2 and encodes hepatocyte nuclear factor 1-alpha *(HNF1A*; OMIM 142410). It is also known as TCF1 (transcription factor 1). HNF1A is a transcription factor that regulates expression of the insulin gene and other genes encoding proteins involved in glucose transport and metabolism. *MODY3* mutations may contribute to abnormal pancreatic islet cell development during fetal life, as well as impaired transcriptional regulation of genes involved in normal islet cell function. *MODY3* mutations also influence expression of *HNF4A* (*MODY1*). This suggests that the MODY transcription factors form a regulator network that is necessary to maintain glucose homostasis.

MODY3 mutations may also contribute to the development of T1D and T2D. Approximately 100 genetic variants of *MODY3* have been identified. These include frameshift, and missense mutations. *MODY3* accounts for the majority (~65%) of MODY cases.



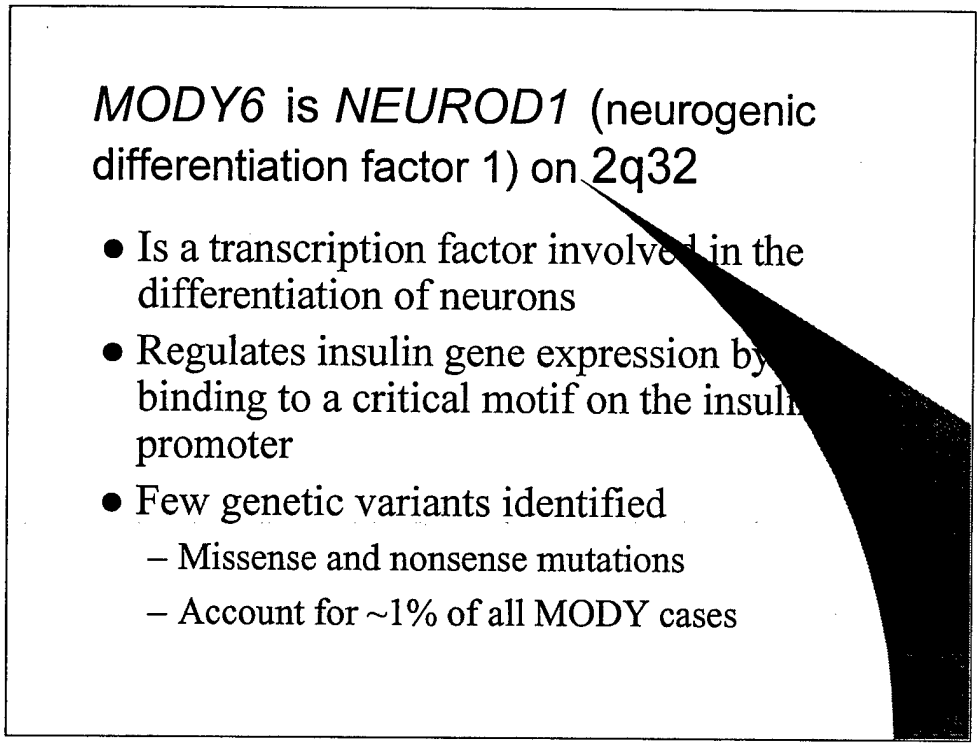
MODY4 is caused by mutations in the insulin promoter factor-1 (*IPF1*; OMIM 600733) gene on chromosome 13q12.1. *IPF1* is a pancreatic transcription factor that regulates expression of insulin, somatostatin and other genes. In addition, *IPF1* is involved in the development of the pancreas. In adults, the expression of *IPF1* is restricted to cells of the pancreas. Individuals with *MODY4* mutations have decreased binding activity to the insulin gene promoter, which leads to reduced activation of the insulin gene in response to glucose.

MODY4 mutations have also been associated with T2D. At least 6 genetic variants have been identified, including frameshift, insertions and missense mutations. They contribute to ~1% of all MODY cases.



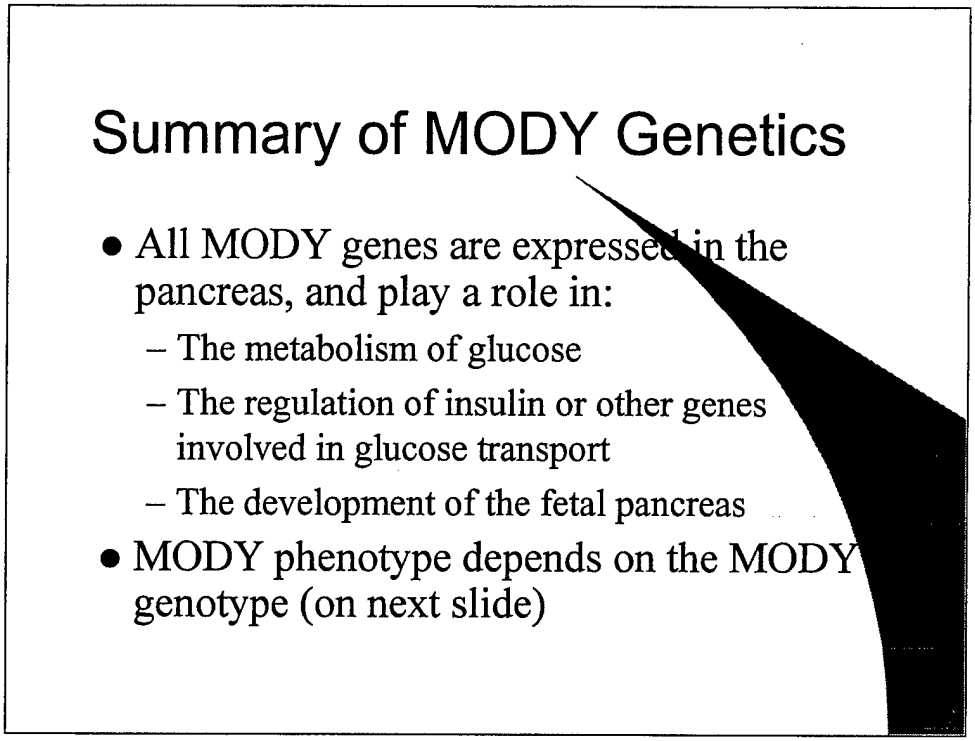
Mutations in the hepatocyte nuclear factor 1-beta gene (HNF1B; OMIM 604284) on chromosome 17cen-q21.3 represent MODY5. This protein is also known as transcription factor 2 (TCF2). *HNF1B* is highly homologous to *HNF1A* (*MODY3*), and appears to *MODY5* mutations have a lower renal threshold to glucose, which predisposes to severe renal disease.

MODY5 mutations (including deletions, missense and nonsense mutations), are a rare cause of MODY and account for ~1% of all MODY cases,



The neurogenic differentiation factor 1 gene (NEUROD1; OMIM 601724) on chromosome 2q32 is *MODY6*. This gene is involved in the differentiation of neurons. It regulates insulin gene expression by binding to a critical motif on the insulin promotor.

MODY6 mutations are a rare cause of MODY. Only a few genetic variants, including missense and nonsense mutations have been identified.



To summarize, all MODY genes are expressed in the islet cells of the pancreas, and play a role in the metabolism of glucose, the regulation of insulin or other genes involved in glucose transport, and / or the development of the fetal pancreas. Because MODY phenotypes vary depending on which MODY gene is involved, genetic testing may also assist in the treatment of the disease.

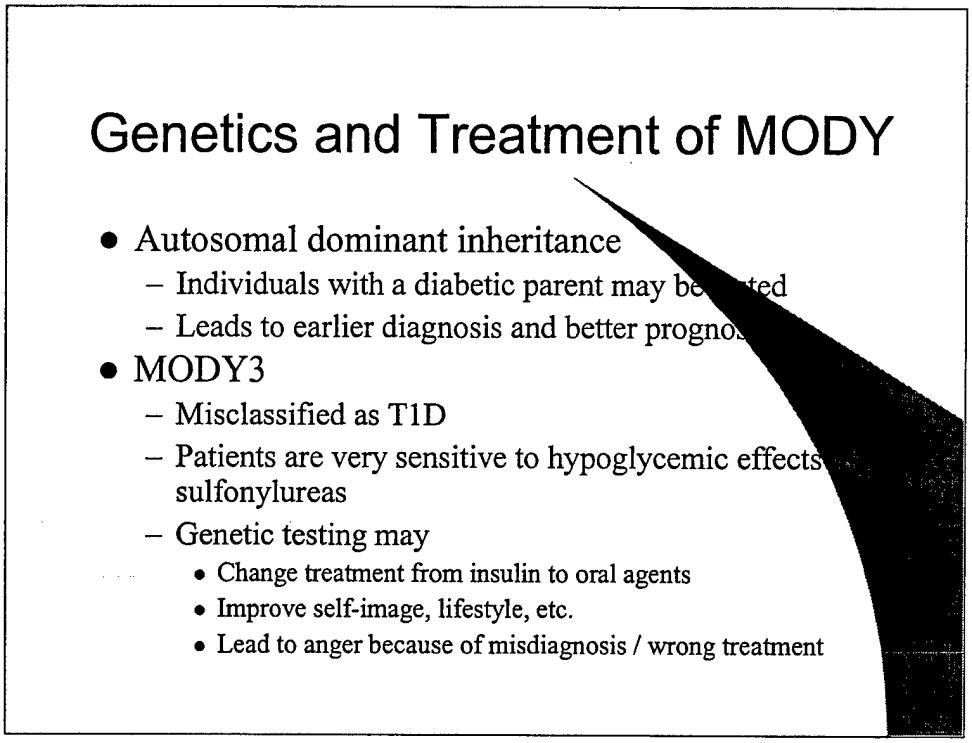
MODY Phenotpes

Type	Onset	Complications	Treatment
MODY1	Severe	Frequent	D, O
MODY2	Mild	Rare	D
MODY3	Severe	Frequent	D, O, I
MODY4	Moderate	Little data	O, I
MODY5	Severe	Renal disease	O, I
MODY6	Severe	Little Data	D, O, O

D = Diet, O = Oral agents, I = Insulin

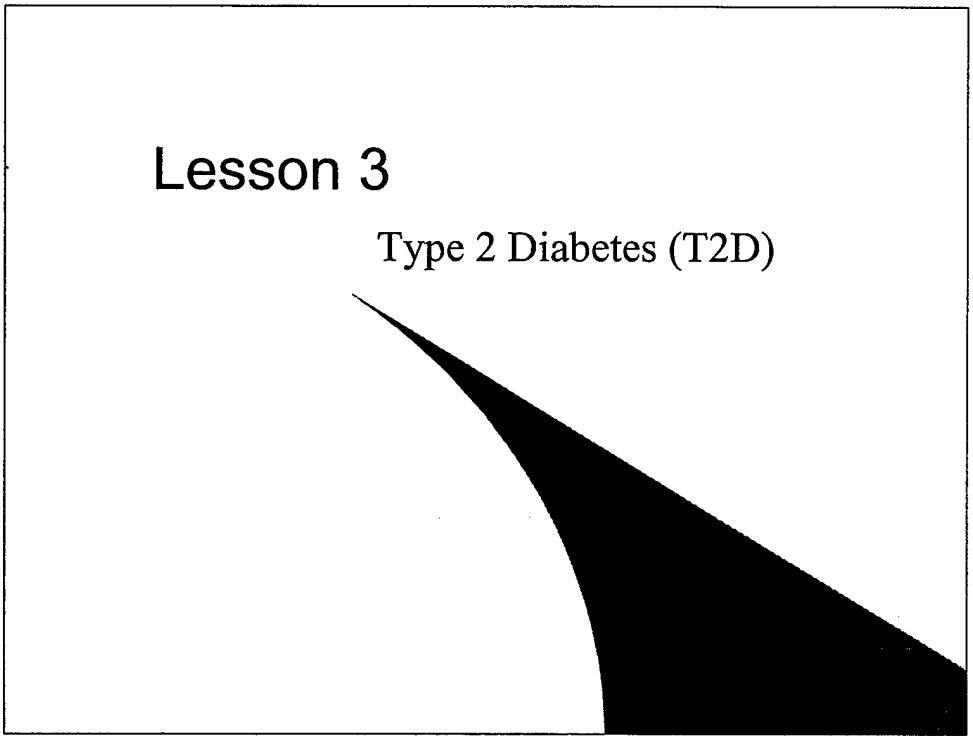
The most common causes of MODY are related to mutations in *MODY1, MODY2 and MODY3*. Although individuals how carry MODY2 mutations have a mild form of the disease, thow who carry MODY1 and MODY3 variants have a much more severe expression that is also associated with long-term complications that develop in persons with other forms of diabetes.

There also has been a link between *MODY3* and *MODY5* because of their interaction in terms of gene expression. However, it is now becoming clear that the metabolic phenotype of individuals with these two forms of the disease is actually quite different. To date, little has been known about *MODY5* other than its association with renal cysts. However, it now appears that *MODY5* is more often characterized by hyperinsulinemia and dyslipidemia (and more closely related to insulin resistance) than *MODY3*. Thus, knowledge about the underlying MODY defect is likely to lead to better management and an improved prognosis for individuals with the disease.

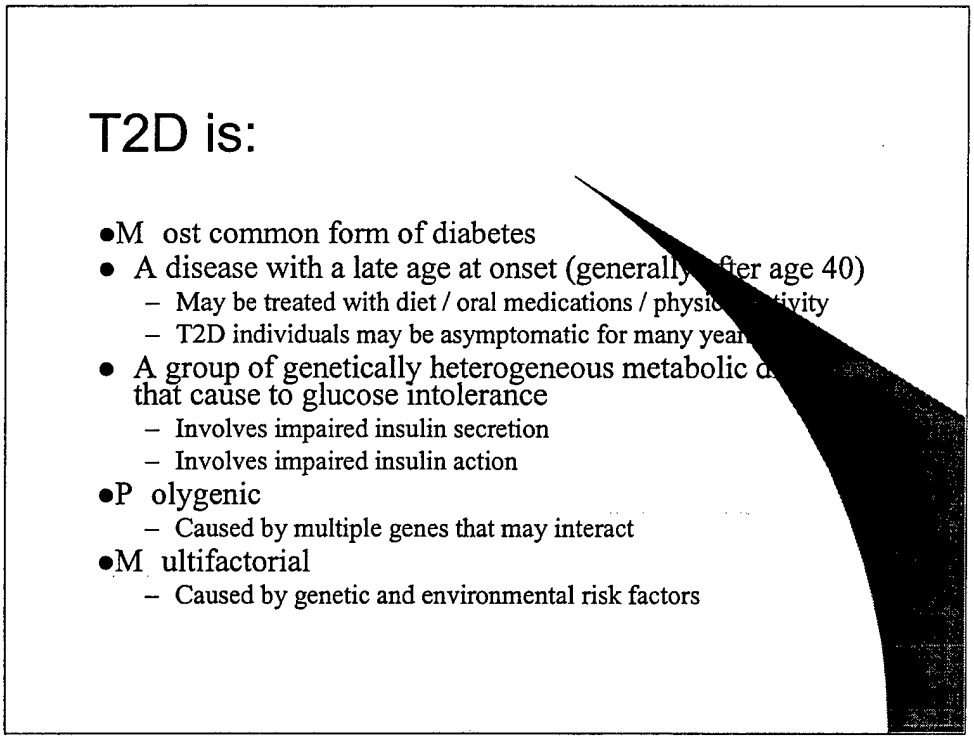


Given the autosomal dominant inheritance of all forms of MODY, individuals with a diabetic parent may wish to have genetic testing. Early diagnosis of MODY may help guide treatment and reduce the likelihood of long-term complications in the severe forms of the disease. In addition, psychological and family adjustments to diabetes may also be improved when the specific form of the disease is known.

Approximately 1/3 of individuals with *MODY3* are treated by diet, oral agents, and insulin. Some individuals with *MODY3* have been previously been characterized as having T1D because of the severity of the disease. It is now know that individuals with *MODY3* are extremely sensitive to the hypoglycemic effects of sulfonylureas. Thus, these oral agents are likely to be the treatment of choice for individuals with ***MODY3***. Recently, there have been a number of reports of *MODY3* individuals being able to change treatment regimens from insulin injections to oral sulphonylurea agents, with considerable improvement in glycemic control. This is frequently associated with a positive impact on lifestyle and self image, as well as fear and anxiety about the possibility of stopping insulin. Some individuals, particularly those with long-term diabetes complications, have become angry because they were previously misdiagnosed and / or treated inappropriately. These reactions have implications for health professionals who need to be knowledgeable about the potential psychological consequences of changing treatment regimens.



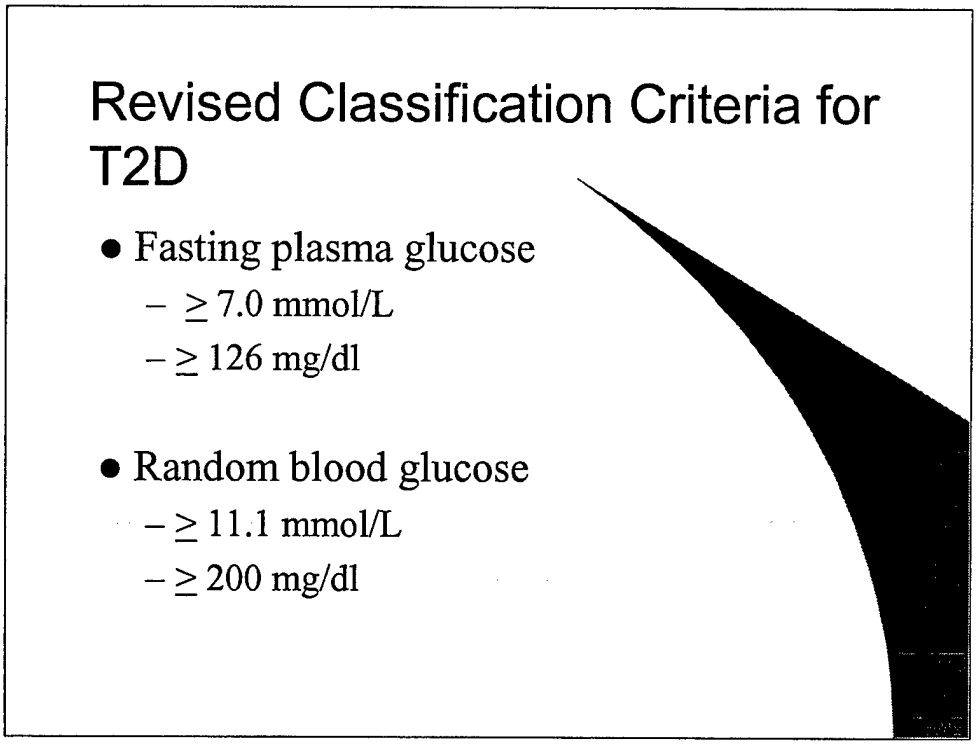
The third section of Module 2 will focus on type 2 diabetes (T2D).



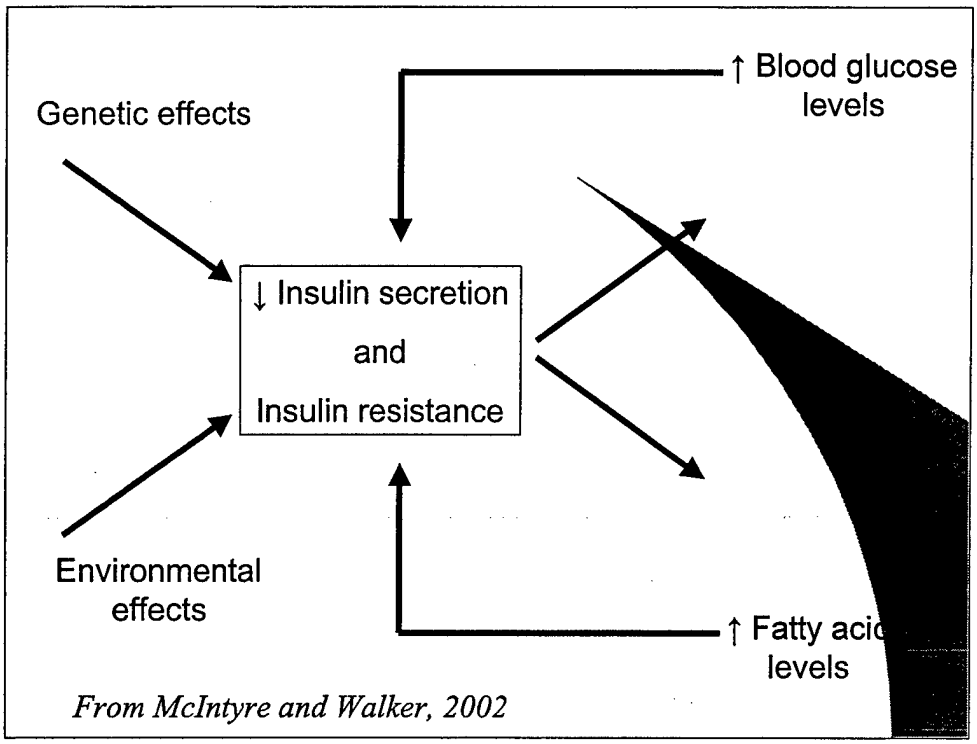
T2D is the most common form of the disease, and is associated with a late age of onset (generally after age 40 years). T2D can often be treated with diet and / or oral hypoglycemic drugs. Insulin therapy may be used, but it is not necessary for survival. As a result, T2D may be relatively asymptomatic for many years before diagnosis.

T2D is considered to be a group of genetically heterogeneous metabolic diseases that ultimately result in glucose intolerance. Individuals with T2D generally have impaired insulin secretion and / or impaired insulin action. Both types of impairments ultimately lead to glucose intolerance and increased levels of circulating fatty acids.

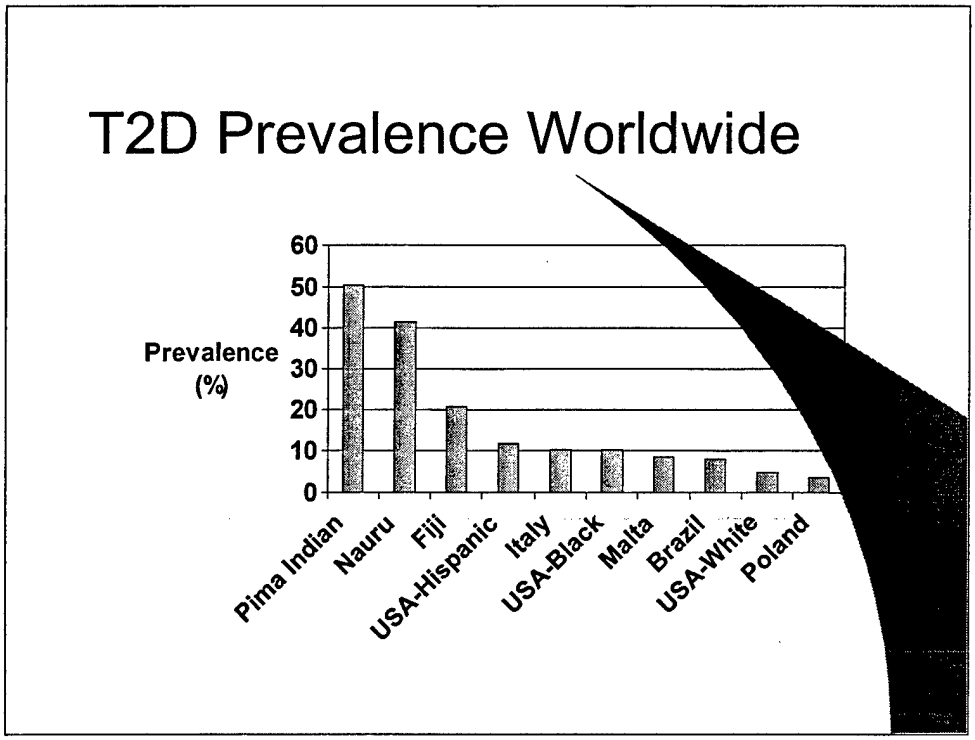
T2D is likely caused by defects in multiple of genes, each of which are appears to have a small effect. However, the interaction of these genes ultimately results in the onset of the disease. In addition to being polygenic, T2D is also a multifactorial disese. Environmental risk factors, particularly obesity and lack of physical activity, contribute to the development of T2D.



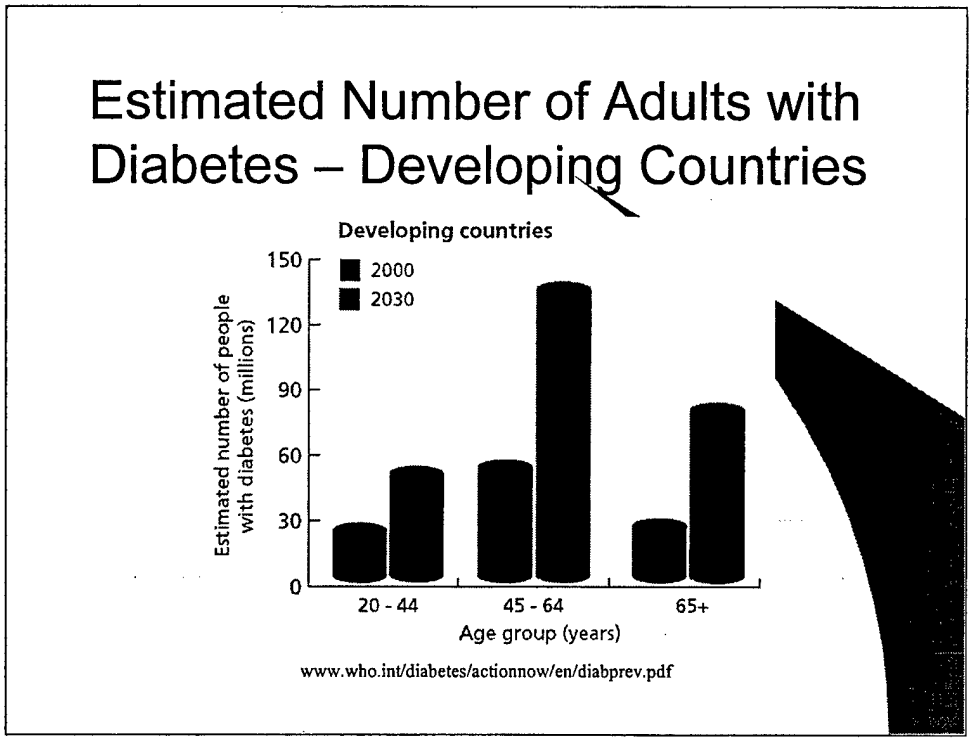
According to the revised classification criteria, a diagnosis of T2D is made if a fasting plasma glucose is ≥ 7.0 mmole/L (≥ 126 mg/dl), or a random blood glucose is ≥ 11.1 mmol/L (≥ 200 mg/dl)



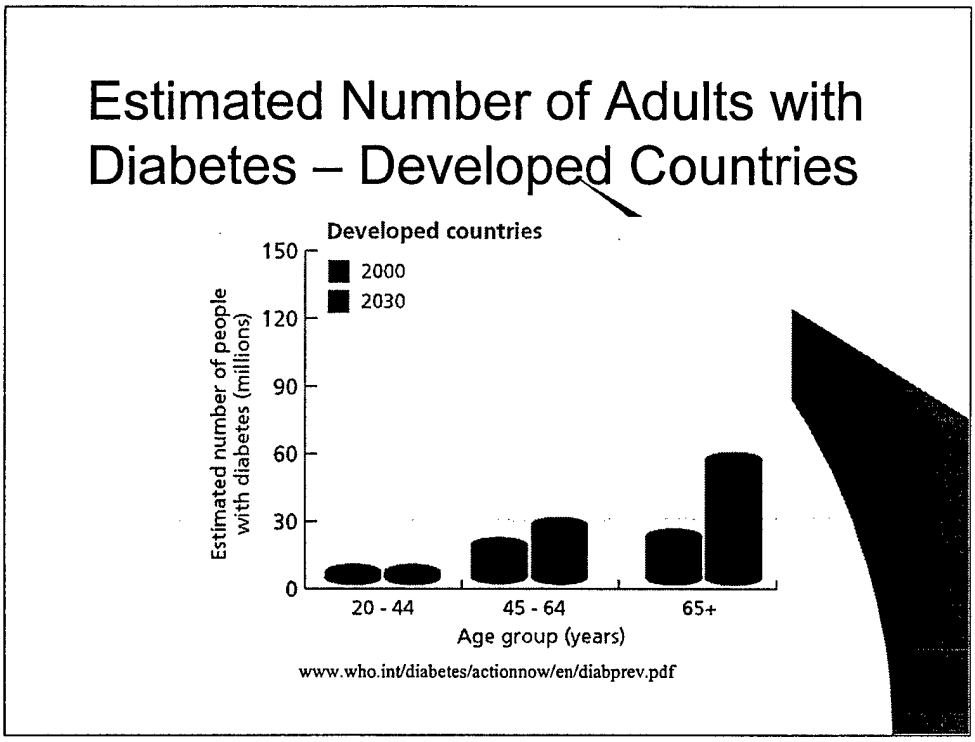
The metabolic consequences of impaired insulin secretion and insulin action feed back and influence levels of blood glucose and fatty acid, which then exert adverse effects on both insulin secretion and insulin action. This complexity has made it extremely difficult to establish direct links between the metabolic changes and their underlying genetic variants.



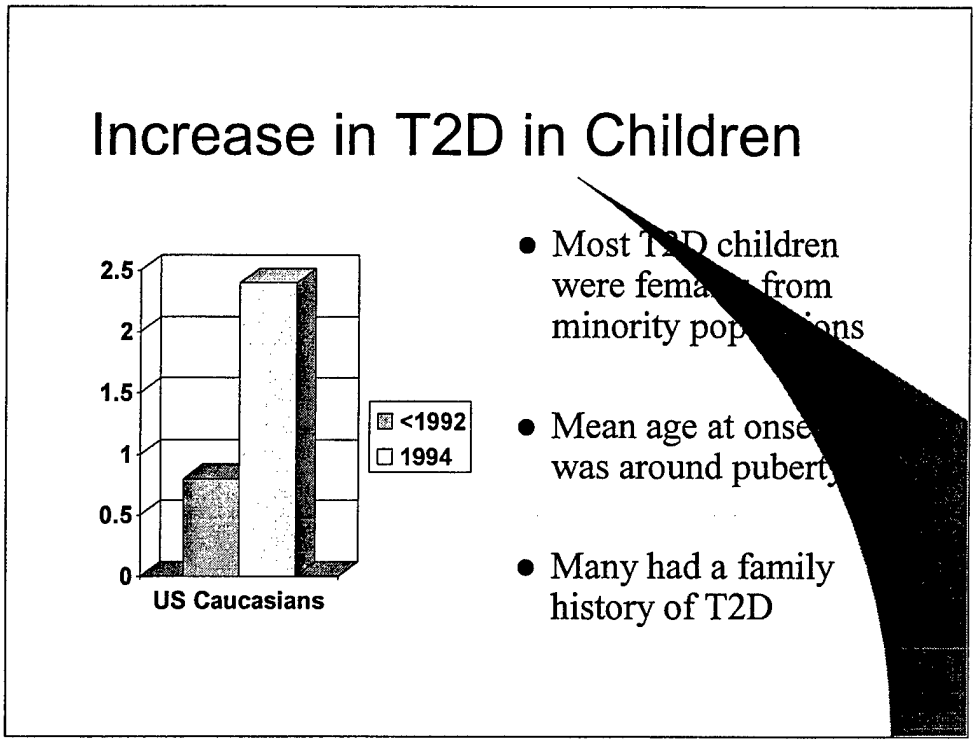
This figure illustrates geographic differences in the prevalence of T2D. The highest prevalence rates are seen in the Pima Indians in the South Western United States, the Nauru in Micronesia, and Fiji. Worldwide, prevalence rates are similar for men and women, although they are slightly higher in men less than age 30 years and in women older than age 65 years.



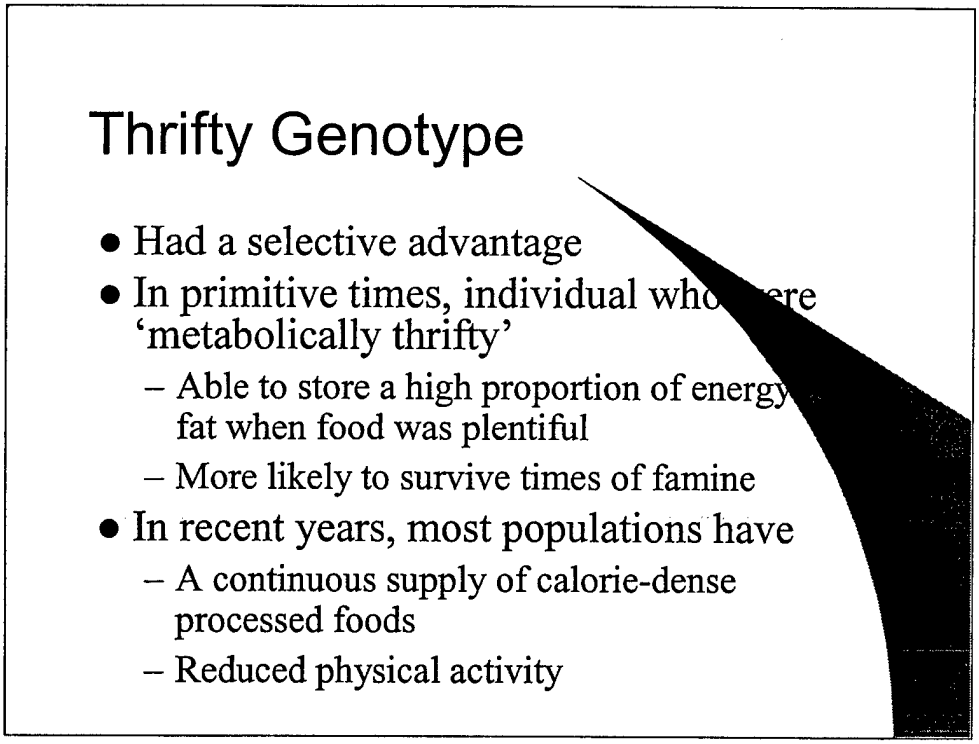
This figure illustrates the number of adults in developing countries with diabetes in the year 2000, and projections for 2030. The number of individuals with diabetes in all age groups is predicted to more than double by 2030, with the largest proportion in the 45-64 year age group. It is anticipated that the majority of newly diagnosed T2D individuals will come from India, China, Indonesia, Pakistan, Brazil, Bangladesh, Japan, Philippines, and Egypt.



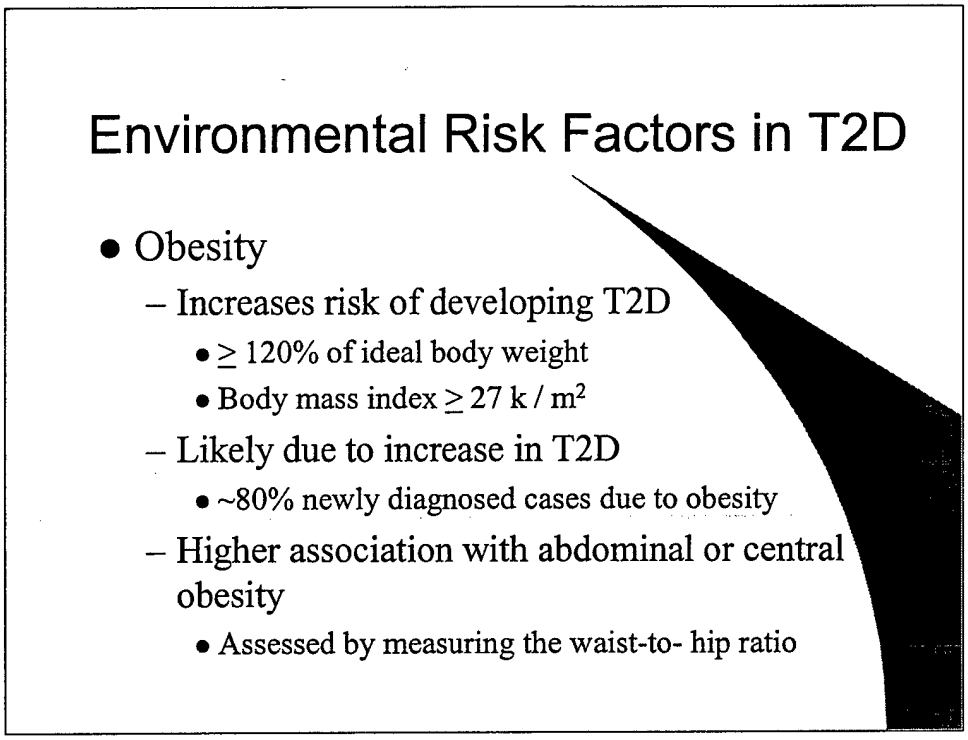
This figure estimates the number of people with diabetes from developed countries, most of whom represent the US. As illustrated, the largest increase is anticipated to be among individuals over age 65 years, which is different from the situation expected for developing countries.



Of great concern is the recent increase in T2D in children. A report based on the Pima Indians in Arizona noted that between 1967-1976 and 1987-1996, the prevalence of T2D increased 6-fold in adolescents (data not shown). In the US, the incidence of T2D increased from 0.3 – 1.2 / 100,000 per year before 1992 to 2.4 / 100,000 per year in 1994. Most T2D children diagnosed during this period were females from minority populations, with a mean age at onset at round puberty. They were also likely to have a positive family history of the disease, particularly maternal diabetes.

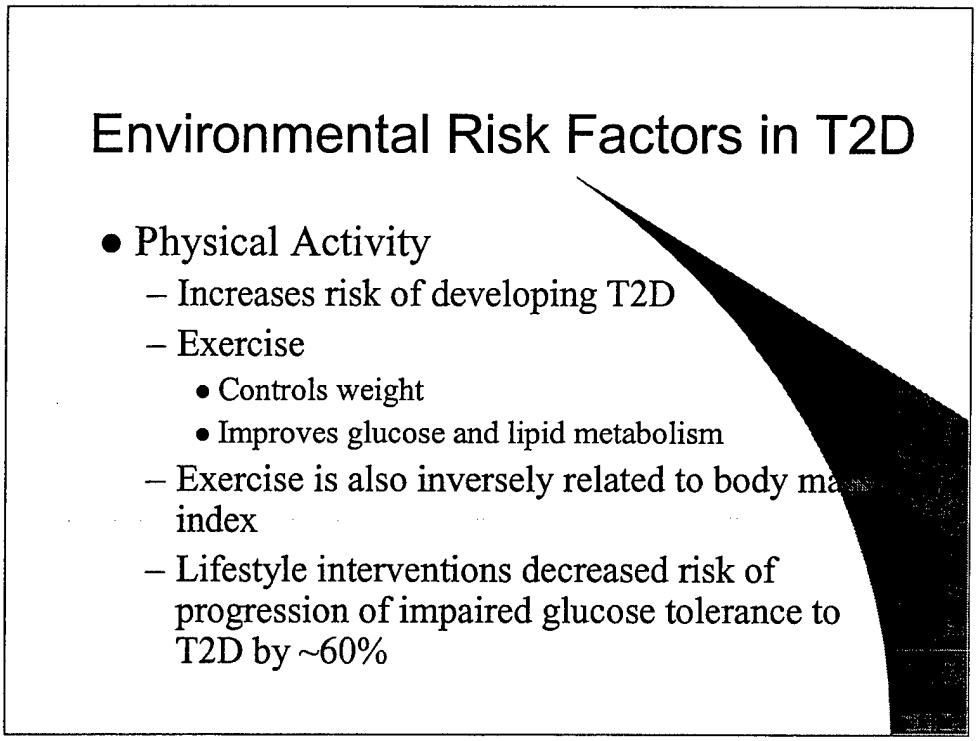


As early as 1962, Neal hypothesized that T2D represented a 'thrifty genotype', which had a selective advantage. He postulated that in primitive times, individuals who were 'metabolically thrifty' and able to store a high proportion of energy as fat when food was plentiful were more likely to survive times of famine. However, in recent years, most populations have a continuous supply of calorie-dense processed food, as well as a decline in their physical activity. This likely explains the rise in T2D prevalence worldwide.



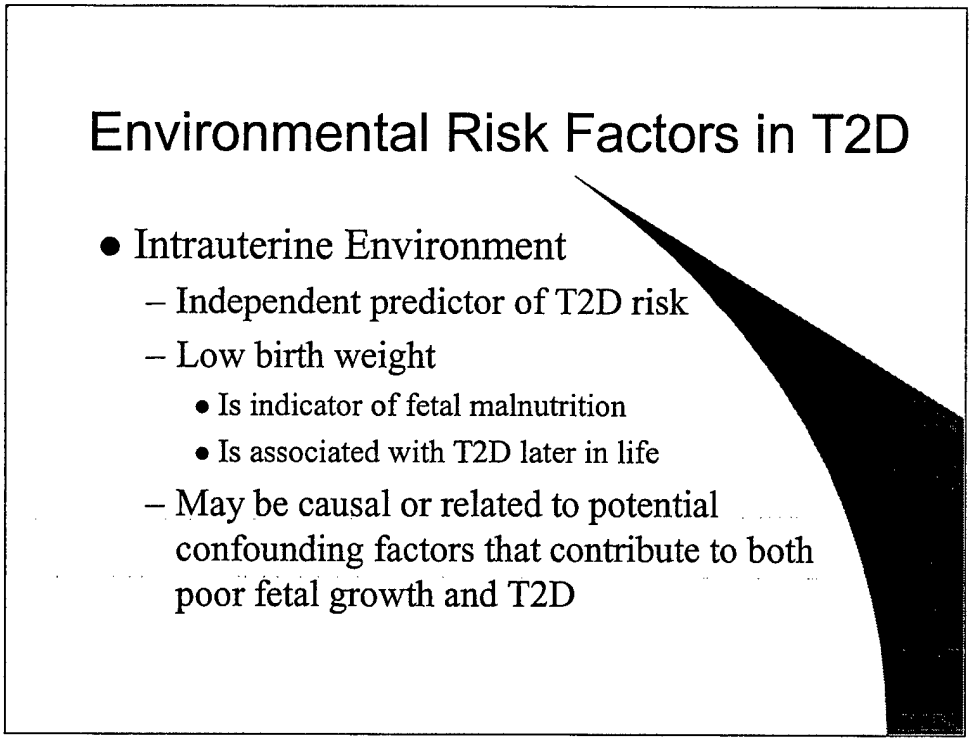
A major environmental risk factor for T2D is obesity, defined by ≥ 120% ideal body weight or a body mass index ≥ 27 k/m^2. Thus, the tremendous increase in the rates of T2D in recent years has been attributed, primarily, to the dramatic risk in obesity worldwide. It has been estimated that approximately 80% of all new T2D cases are due to obesity. This is true for children and adults.

In addition to general obesity, the distribution of body fat, estimated by the ratio of the waist-to-hip circumferences (WHR). WHR is a reflection of abdominal (central) obesity, which is more strongly associated with T2D than standard measures, such as the body mass iodex.

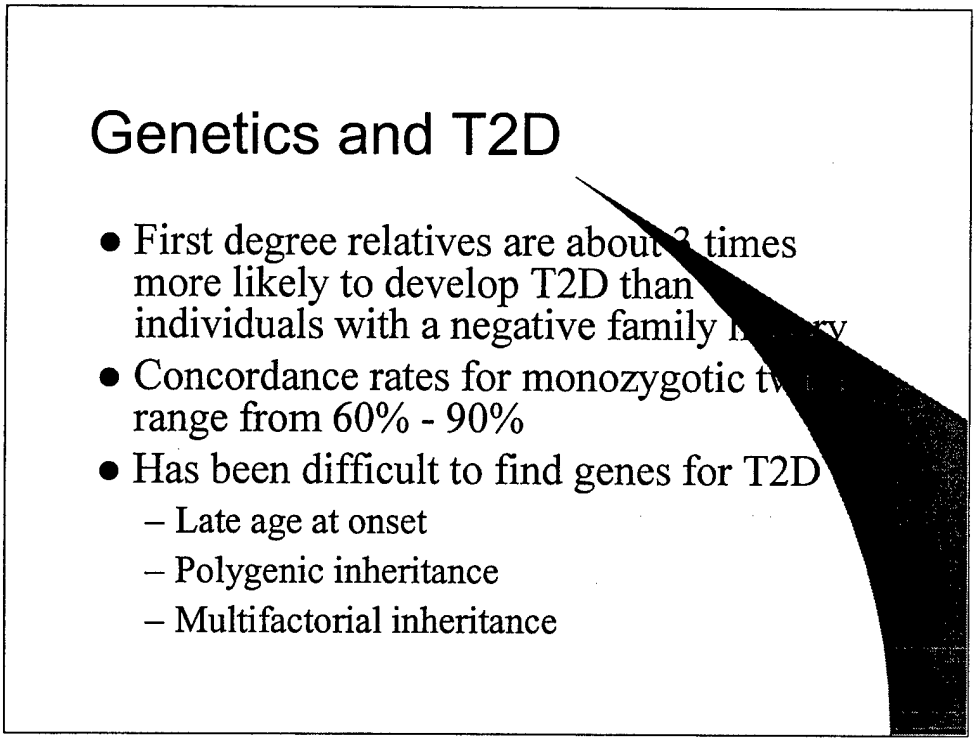


The other major T2D risk factor is physical inactivity. In addition to controlling weight, exercise improves glucose and lipid metabolism, which decreases T2D risk. Physical activity, such as daily walking or cycling for more than 30 minutes, has been shown to significantly reduce the risk of T2D. Physical activity has also been inversely related to body mass index.

Recently, intervention studies in China, Finland, and the US have shown that lifestyle interventions targeting diet and exercise decreased the risk of progression of impaired glucose tolerance to T2D by approximately 60%. In contrast, oral hypoglycemic medication only reduced the risk of progression by about 30%.

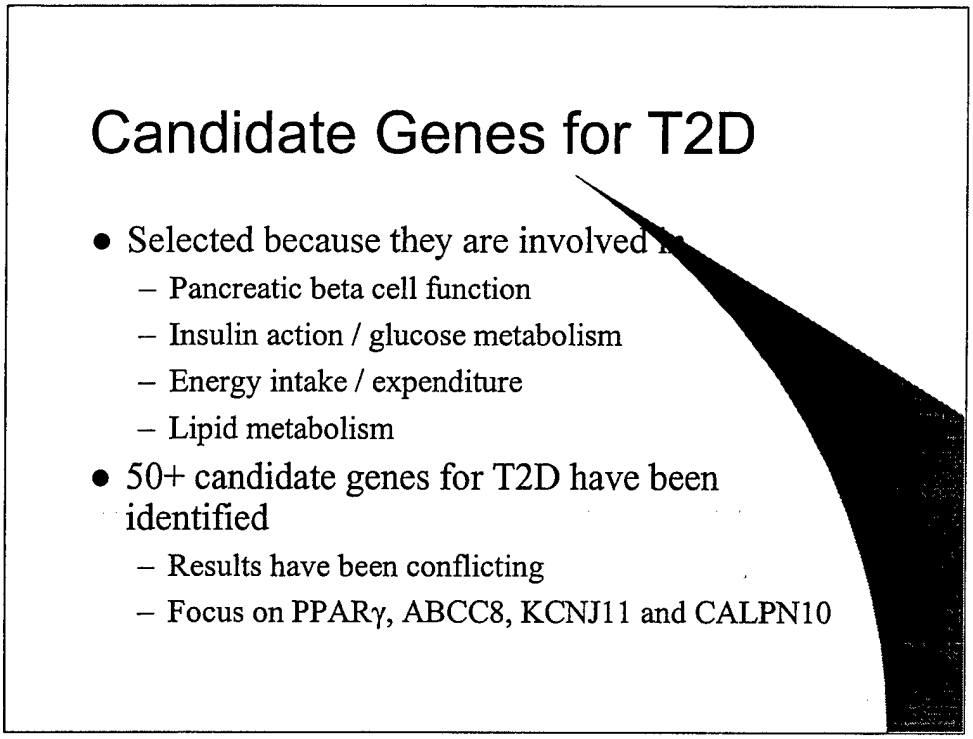


There is also considerable evidence suggesting that the intrauterine environment is an important predictor of T2D risk. Numerous studies have shown that low birth weight, which is an indicator of fetal malnutrition, is associated with T2D later in life. However, it is unclear whether low birth weight is causal or related to potential confounding factors that contribute to both poor fetal growth and T2D.

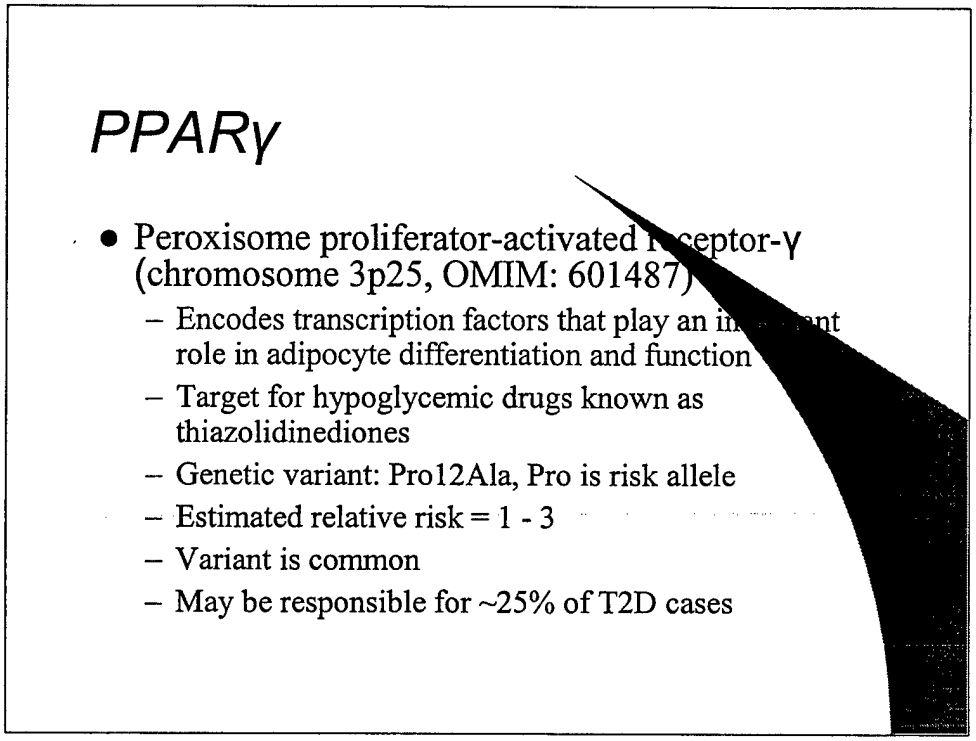


It has long been known that T2D is, in part, inherited. Family studies have revealed that first degree relatives of individuals with T2D are about three times more likely to develop the disease than individuals without a positive family history of the disease. It has also been shown that concordance rates for monozygotic twins, which have ranged from 60% - 90%, are significantly higher than those for dizygotic twins. Thus, it is clear that T2D has a strong genetic component.

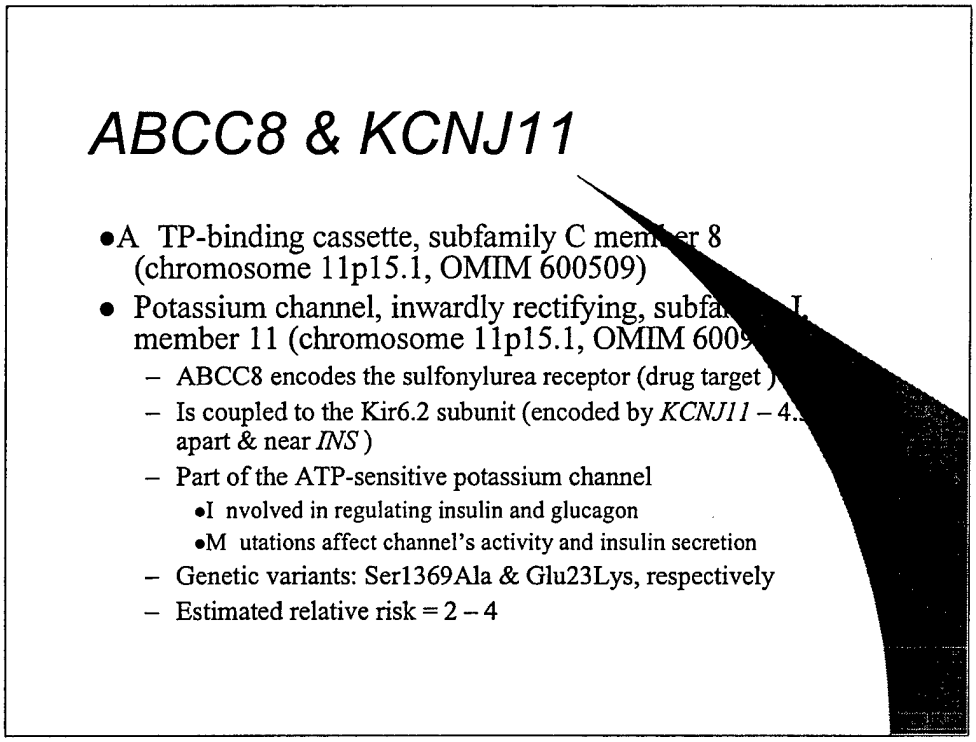
However, it has been difficult to find genes that increase T2D risk for a number of reasons. First, the disease has a late age at onset. Thus, individuals who will develop T2D in the future may be asymptomatic at the time they are studied. As previously indicated, T2D is caused by multiple genes, each of which are likely to have a small effect. In addition, environmental factors contribute to the development of T2D – and these may vary across families and populations.



One approach that is used to identify disease susceptibility genes is based on the identification of candidate genes. Candidate genes are selected because they are thought ot be involved in pancreatic beta cell function, insulin action / glucose metabolism, or other metabolic conditions that increase T2D risk (e.g., energy intake / expenditure, lipid metabolism). To date, more than 50 candidate genes for T2D have been studied in various populations. However, results for essentially all of the them has been conflicting. Possible explanations for the divergent findings include small sample sized, differences in T2D susceptibility across ethnic groups, variations in environmental exposures, and gene-gene interactions. Because of the current controversy, we will discuss only a few of the most promising candidate genes. These include PPARγ, ABCC8, KCNJ11 and CALPN10.



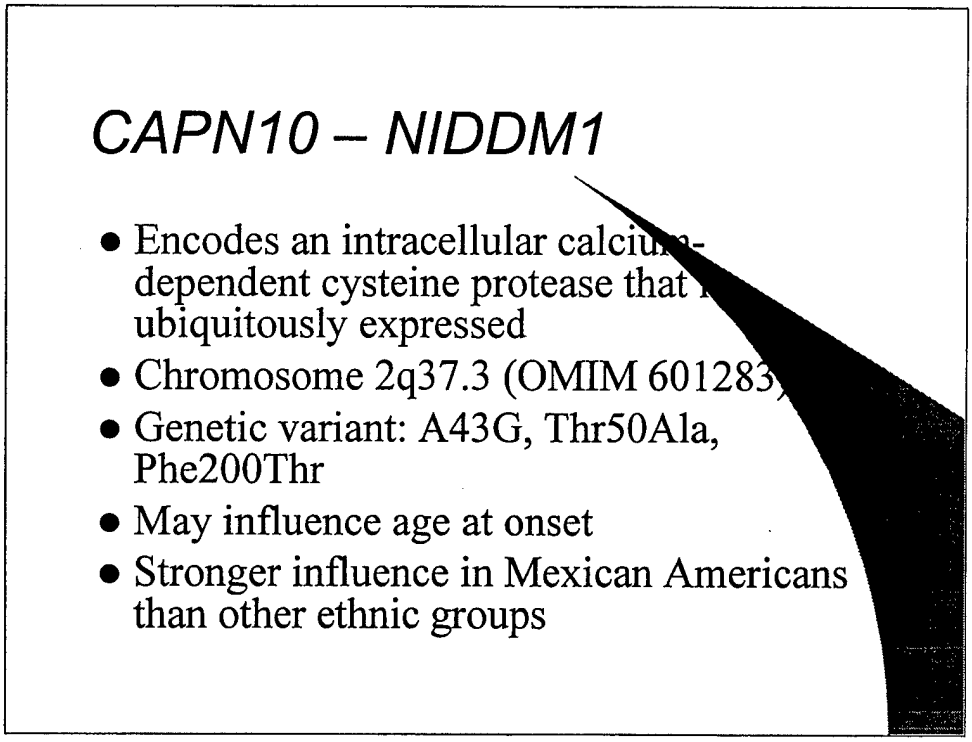
PPARγ has been widely studied because it is important in adipocyte and lipid metabolism. In addition, it is a target for the hypoglycemic drugs known as thiazolidinediones. One form the *PPARγ* gene (Pro) decreases insulin sensitivity and increases T2D risk by several fold. Perhaps most importantly is that this variant is very common in most populations. Approximately 98% of Europeans carry at least one copy of the Pro allele. Thus, it likely contributes to a considerable proportion (~25%) of T2D that occurs, particularly in Caucasian populations.



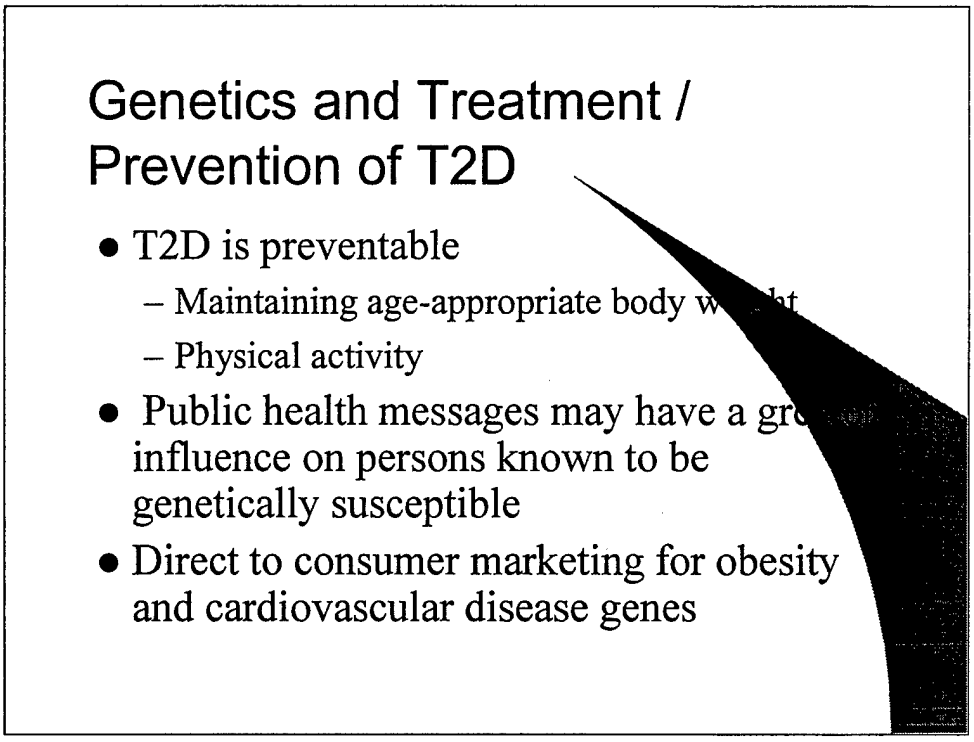
ABCC8 encodes a high affinity sulphonylurea receptor *SUR1* that is coupled to the Kir6.2 subunit, which is encoded by *KCNJ11*. Both genes are part of the ATP-sensitive potassium channel, which plays a key role in regulating the release of hormones such as insulin and glucagon in the beta cell. Mutations in either gene can affect the potassium channel's activity and insulin secretion, ultimately leading to the development of T2D. Interestingly, ABCC8 and KCNJ11 are only 4.5 kb apart, and not far from the INS gene. Variant forms of KCNJ11 (Lys) and ABCC9 (ala) genes have been associated with T2D, as well as other diabetes-related traits. Because of the close proximity of these genes, current studies are evaluating whether they work in concert with each other, or rather have na independent effect on T2D susceptibility.

T2D Genes are Drug Targets

- PPARγ, ABCC8 and KCNJ11 are the targets of drugs used routinely in the treatment of T2D
  - Pharmacogenetic implications
  - Response to oral agents may be related to one's genotype
  - Genetic testing may
    - Identify individuals at high risk for T2D
    - Guide treatment regimens for T2D

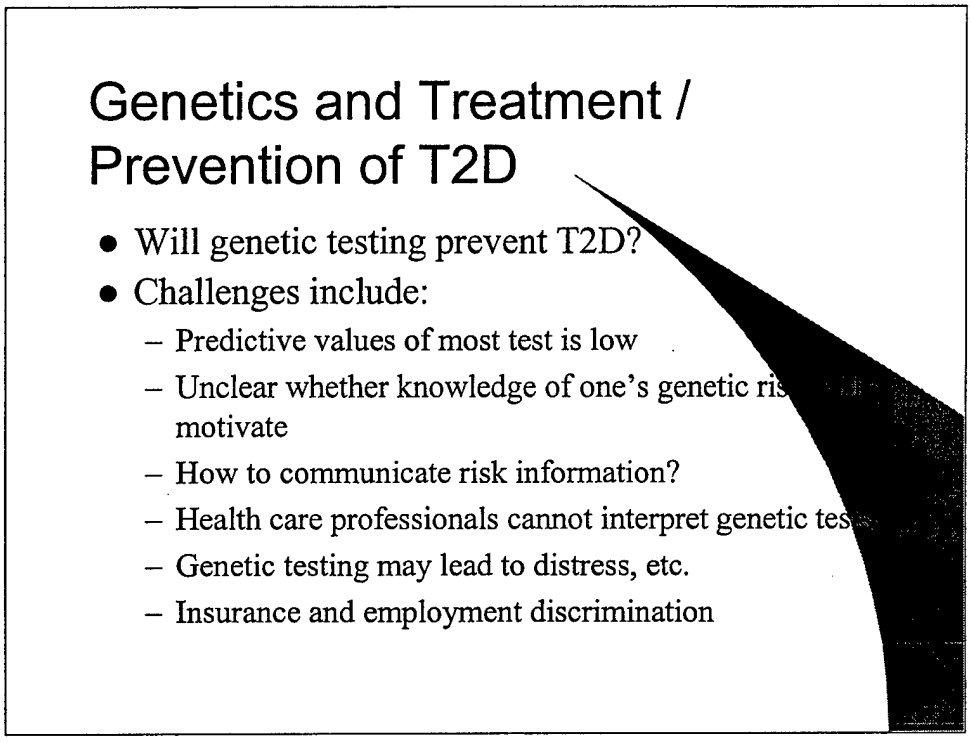


CAPN10, now known as *NIDDM1*, was originally mapped to the tip of the long arm of chromosome 2 in Mexican American sib-pairs. It encodes an intracellular calcium-dependent cysteine protease (calpain 10) that is ubiquitously expressed. A haplotype that was initially linked to T2D included an intronic A to G mutation at position 43, which appears to be involved in *CAPN10* transcription. Two amino acid polymorphisms (Thr504Ala and Phe200Thr) have also been associated with T2D risk. However, it has been suggested that the coding and nonconding polymorphisms do not independently influence T2D risk, but instead contribute to an early age at diagnosis. Physiological studies suggest that variations in calpain 10 activity effects insulin secretion, and therefore, susceptibility to T2D. Studies from other ethnic groups indicate that the contribution of this locus to T2D risk may be much larger in Mexican-American than Caucasian populations.

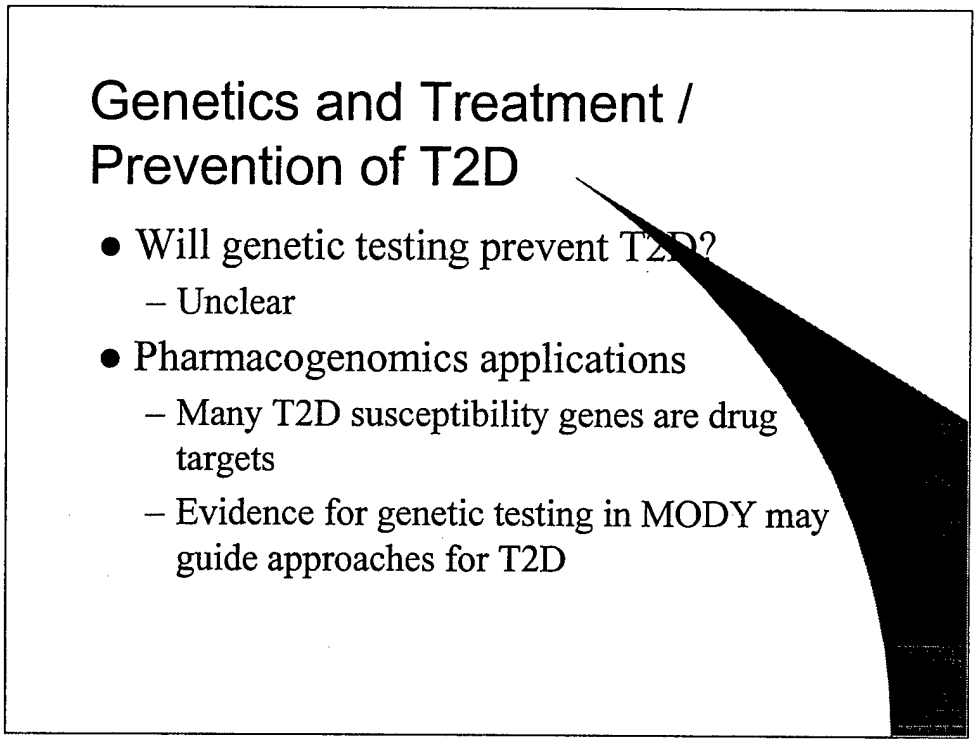


Unlike T1D and MODY, T2D can generally be prevented by maintaining an age-appropriate body weight and engaging in physical activity. Given the recent obesity epidemic, it is obvious that current intervention strategies are being ignored by a majority of individuals in the general population.

Leaders of the Human Genome Project have predicted that genetic tests for many common diseases would be available in first decade of the 21st century, permitting persons 'to learn their individual susceptibilities and to take steps to reduce those risks' by applying interventions based on 'medical surveillance, lifestyle modification, diet or drug therapy' (Collins and McKusick, 2001). In fact several companies are now offering genetic susceptibility testing, which can be ordered online by any individual, for conditions such as cardiovascular disease and obesity.



Some scientists and health professionals doubt that genetic testing will help prevent chronic diseases, such as T2D for a variety of reasons. First, the predictive value of most genetic tests is low, and risk estimates do not account for well-known environmental determinants of the disease. Secondly, it is unclear whether knowledge about one's genetic risk will increase motivation to engage in disease interventions. Thirdly, genetic testing presents education and information-dissemination challenges that were outlined in detail by the Secretary's Advisory Committee on Genetics, Health and Society. Fourthly, most health professionals are currently unqualified to interpret the results of genetic tests. Fifthly, genetic testing may lead to significant distress, the magnitude of which is likely to vary as a function of test results, coping skills, risk perception and other characteristics. These factors relate directly to other concerns such as insurance and employment discrimination, confidentiality and stigmatization based on knowing that one is at high genetic risk.



In the near future, genetic testing for T2D and other chronic diseases will most certainly become available. Although it is unclear wither this will actually contribute to the prevention of T2D, it may be beneficial in terms of disease management. Many of the current T2D susceptibility genes of interest are drug targets. Evidence for the role of pharmacogenomics in diabetes is already apparent in treatment approaches for MODY.

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ANNUAL REPORT

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APPENDIX 2:

GIFT-D ADDITIONAL MATERIAL

ABSTRACTS AND MANUSCRIPTS

National Institute of Nursing Research

Linking The Double Helix With Health: Genetics in Nursing Research

POSTER SESSION ABSTRACTS

April 13th, 2003
Georgetown University School of Nursing and Health Studies

Designing an Interactive Website for Nurses to Meet the Genetic Education Needs of Patients and Their Families

Presenter: Angela Feathers, Genetic Counseling Graduate Student, University of Pittsburgh, Graduate School of Public Health, Department of Human Genetics

The vast amount of knowledge generated by the Human Genome Project has accelerated efforts to elucidate the hereditary components of complex diseases including cancer and diabetes. Such research endeavors have the potential to revolutionize standard approaches to diagnosis, treatment, and prevention of diseases. Therefore, a fundamental understanding of genetics is becoming increasingly important for nurses, patients and their families. By developing a working knowledge of genetics, nurses can play an integral role in informing and educating patients about advances in genetics and disease risk. Although genetic counselors have traditionally assumed this role, rapid developments in medical genetics demand that nurses begin to function as part of multidisciplinary teams to promote patients' comprehension of genetics in relation to disease. Computer-based resources for genetics education that are targeted towards the general population can serve as valuable tools for nurses as they explain genetic concepts to individuals confronting multifactorial disorders. To address this critical need for genetic literacy, we are designing an interactive web-based program for nurses to educate type 1 diabetes (T1D) families about the genetics of the disease. This novel educational initiative will hopefully serve as a model for developing future programs related to other complex diseases. Ultimately, incorporation of these resources into standard health care will help nurses translate relevant genetic information to patients, their families, and the public.

Internet Based Education for Nurses

From Research to Practice

Eric R. Manthei, B.S., Genetic Counseling Student; Angela Feathers, B.S., Genetic Counseling Student; Elizabeth (Betsy) Gettig, M.S., C.G.C.; Linda Siminerio, R.N., Ph.D.; Janice Dorman, Ph.D.

Abstract

The Human Genome Project has made genetics a household word and, therefore, has challenged current nursing practice. These challenges can be met, in part, by integrating research tools into clinical practice. For example, interactive web-based genetic education modules are being developed to train research nurses involved in genetic studies of chronic diseases. We are developing just such an approach for type 1 diabetes (T1D) because considerable research, including genome-wide screens, is focusing on high-risk families recruited from clinical settings by genetic screening. Research nurses typically coordinate these studies, and therefore need additional training regarding the genetics of the disease. Our genetic education program consists of 3 web-based modules covering: 1) basic human genetics, 2) the genetics of T1D, and 3) the processes of genetic risk evaluation and risk communication. Continuing nursing education credits can also be obtained. Although our web-based program is specific for T1D it has broad applications to other fields of nursing research. Modules 2 and 3 could easily be modified for other specific disorders, which could train research nurses conducting other studies that involve genetic testing. However, the program is also relevant to nursing practice. Clinical nurses and other health professionals could also be trained by using such web-based programs as this would enhance the genetic expertise of a clinical team. Internet-based genetic education programs to train nurses are becoming increasingly important as the Human Genome Project continues to revolutionize nursing research, as well as the practice of medicine.

Genetics and Diabetes

Janice S. Dorman, Ph.D., University of Pittsburgh, School of Nursing, 350 Victoria Building, Pittsburgh, PA 15261, Phone: 412-624-8392, FAX: 412-624-2401, Email: jsd@pitt.edu.

Background

Diabetes mellitus is a heterogeneous group of disorders characterized by persistent hyperglycemia. The two most common forms of diabetes are type 1 diabetes (T1D, previously known as insulin-dependent diabetes or IDDM) and type 2 diabetes (T2D, previously known as non-insulin-dependent diabetes or NIDDM). Both are caused by a combination of genetic and environmental risk factors. However, there are other rare forms of diabetes that are directly inherited. These include maturity onset diabetes in the young (MODY), and diabetes due to mutations in mitochondrial DNA.

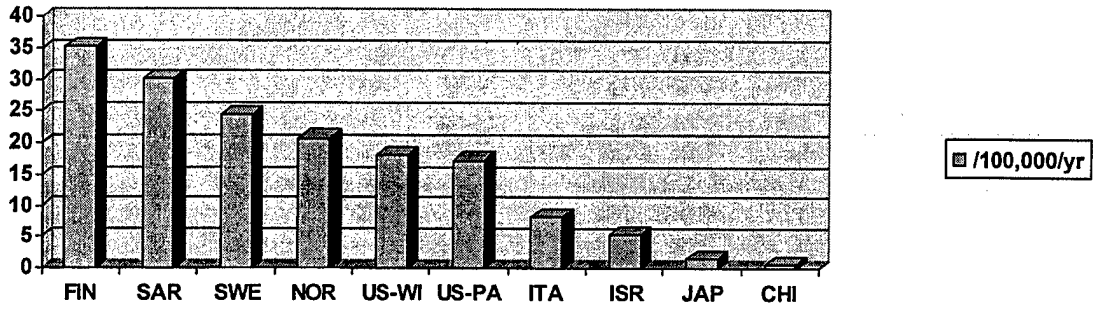
All forms of diabetes have very serious effects on health. In addition to the consequences of abnormal metabolism of glucose (e.g., hyperlipidemia, glycosylation of proteins, etc.), there are a number of long-term complications associated with the disease. These include cardiovascular, peripheral vascular, ocular, neurologic and renal abnormalities, which are responsible for morbidity, disability and premature death in young adults. Furthermore, the disease is associated with reproductive complications causing problems for both mothers and their children. Although improved glycemic control may decrease the risk of developing these complications, diabetes remains to be a very significant cause of social, psychological and financial burdens in populations worldwide.

Type 1 Diabetes

Epidemiology. T1D is caused by the autoimmune destruction of the beta cells of the pancreas, and represents approximately 10% of all cases with diabetes. At present, lifelong insulin therapy is the only treatment for the disease. Without exogenous insulin injections, individuals with T1D will not survive. Although the prevalence of T1D is <1% in most populations, the geographic variation in

incidence is enormous, ranging from <1/100,000 per year in China to approximately 40/100,000 per year in Finland (Figure 1) (Karvonen et al., 1993). The only chronic childhood disorder more prevalent than T1D is asthma. It has been estimated that approximately 20 million people worldwide, mostly children and young adults, have T1D (Holt, 2004).

Figure 1. T1D Incidence Rates Worldwide



FIN = Finland, SAR = Sardinia, SWE = Sweden, NOR = Norway, US-WI = US-Wisconsin, US-PA = US-Pennsylvania, ITA = Italy, ISR = Israel, JAP = Japan, CHI = China

The incidence of T1D is increasing worldwide at a rate of about 3% per year (Onkamo et al., 1999). This trend appears to be most dramatic in the youngest age groups, and is completely unrelated to the current increase in T2D in children. More children with beta cell autoantibodies, a hallmark of T1D, are being diagnosed with the T1D around the world each year. Although the peak age at onset is at puberty, T1D can also develop in adults. Epidemiologic studies have revealed no significant gender differences in incidence among individuals diagnosed before age 15 (Kyvik et al., 2004). However, after age 25, the male to female incidence ratio is approximately 1.5. There is also a notable seasonal variation in the incidence of T1D in many countries, with lower rates in the warm summer months, and higher rates during the cold winter (Dorman et al., 2003).

Environmental Risk Factors. The epidemiological patterns described above suggest that environmental factors contribute to the etiology of the T1D. In particular, the recent temporal increase in T1D incidence points to a changing global environment rather than variation in the gene pool, which require the passage of multiple generations. Twin studies also provide evidence for the importance of environmental risk factors for T1D. T1D concordance rates for monozygous twins are higher than those for dizygous twins (approximately 30% vs. 10%, respectively) (Hirschhorn, 2003). However, most monozygous twin pairs remain discordant. Thus, T1D cannot be completely genetically determined.

Environmental risk factors are thought to act as either 'initiators' or 'accelerators' of beta cell autoimmunity, or 'precipitators' of overt symptoms in individuals who already have evidence of beta cell destruction. They also may function by mechanisms that are directly harmful to the pancreas, or by indirect methods that produce an abnormal immune response to proteins normally present in cells. The T1D environmental risk factors that have received most attention are viruses and infant nutrition.

Enteroviruses, especially Coxsackie virus B (CVB), have been the focus of numerous ecologic and case-control studies (Dahlquist et al., 1998). CVB infections are frequent during childhood and are known to have systemic effects on the pancreas. Recent prospective studies are helping to elucidate the role of viruses to the etiology of T1D. For example, enteroviral infections occurring as early as *in utero* appear to increase a child's subsequent risk of developing the disease (Dahlquist et al., 1995, Hyoty et al., 1995). Other viruses, including mumps (Hyoty et al., 1993),

cytomegalovirus (Pak et al., 1988), rotavirus (Honeyman et al., 2000) and rubella, (McIntosh and Menser, 1992) have also been associated with the disease.

Another hypothesis that has been the subject of considerable interest relates to early exposure to cow's milk protein and the subsequent development of T1D. The first epidemiologic observation of such a relationship was by Borch-Johnsen et al., who found that T1D children were breast-fed for shorter periods of time than their non-diabetic siblings or children from the general population (Borsh-Johnsen et al., 1984). The authors postulated that the lack of immunologic protection from insufficient breast-feeding may increase risk for T1D later during childhood. It was also postulated that shorter duration of breast feeding may indirectly reflect early exposure to dietary proteins that stimulate an abnormal immune response in newborns. Most recently it has been hypothesized that the protective effect of breast-feeding may be due, in part, to its role in gut maturation (Kolb and Pozzilli, 1999; Harrison and Honeyman, 1999; Vaarala, 1999). Breast milk contains growth factors, cytokines, and other substances necessary for the maturation of the intestinal mucosa. Breast-feeding also protects against enteric infections during infancy, and promotes proper colonization of the gut. Interestingly, enteroviral infections can also interfere with gut immunoregulation, which may explain the epidemiologic associations between viral infections and T1D.

The role of hygiene in the etiology of T1D is also currently being explored (McKinney et al., 1997; Marshall et al., 2004). It has been hypothesized that delayed exposure to microorganisms due to improvements in standard of living hinders the development of the immune system, such that it is more likely to respond inappropriately when introduced to such agents at older (compared to

younger) ages. This explanation is consistent with recent reports indicating that factors such as day care attendance (McKinney et al. 2000), sharing a bedroom with a sibling, and contact with pets are protective against T1D (Marshall et al., 2004). Further studies are needed to determine if improved hygiene can explain the temporal increase in the incidence of T1D worldwide.

Type 2 Diabetes

Epidemiology. T2D is the most common form of the disease, accounting for approximately 90% of all affected individuals. According to the revised classification criteria, a diagnosis of T2D is made if a fasting plasma glucose concentration is $\geq$ 7.0 mmol/L ($\geq$ 126 mg/dl) or a random blood glucose is $\geq$ 11.1 mmol/L ($\geq$ 200 mg/dl) (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 1998). T2D is caused by relative impaired insulin secretion and peripheral insulin resistance. Typically, T2D is managed with diet, exercise, oral hypoglycemic agents and sometimes exogenous insulin. However, it is associated with the same long-term complications as T1D.

The highest rates of T2D are found among Native Americans, particularly the Pima Indians who reside in Arizona in the US, and in natives of the South Pacific islands, such as Nauru (Wild et al., 2004). T2D is also known to be more predominant in Hispanic and African American populations than in Caucasians of European descent. In 2000, the overall prevalence of T2D worldwide was estimated to be 2.8%, but projections for 2030 have almost doubled (4.4%). This corresponds to a worldwide increase from 171 million persons with diabetes in 2000 to 366 million persons in 2030. The greatest relative increase in prevalence is expected to occur in the Middle East, sub-Saharan Africa and India. According to Wild et al., the 'top' three countries in terms of the prevalence of T2D are India (31.7 million in 2000; 79.4 million in 2030), China (20.8 million in 2000; 42.3 million in

2030) and the US (17.7 million in 2000; 30.3 million in 2030). Clearly, T2D has become an epidemic in the 21st century.

In addition to the burden of T2D, it has been estimated that at least 200 million people worldwide have impaired glucose tolerance (IGT) (Wild et al., 2004). Individuals with IGT have higher than normal levels of fasting plasma glucose ($\geq$ 6.1-6.9 mmole/l or $\geq$ 110 - 125 mg/dl), but they are not sufficiently elevated to warrant a diagnosis of T2D. IGT is considered to be a risk factor for the future development of T2D. Approximately 40% of individuals with IGT will go on to develop T2D. They are also more likely to develop the long-term complications associated with T2D compared to persons with normal glucose tolerance.

The prevalence of T2D increases with age (Wild et al., 2004). In developing countries, most individuals with diabetes are between 45 and 64 years of age. In contrast, the greatest prevalence of the disease in developed countries occurs among persons older than age 65 years. Worldwide rates are similar in men and women, although they are slightly higher in men < 60 years of age and in women > age 65 years.

Of great concern is the recent increase in T2D in children (Bloomgarden, 2004). A report based on the Pima Indians in Arizona noted that between 1967-76 and 1987-96, the prevalence of T2D increased 6-fold in adolescents (Fagot-Campagna et al., 2000). In US Caucasians, the incidence of T2D increased from 0.3-1.2/100,000/yr before 1992 to 2.4/100,000/yr in 1994 (Weill et al., 2004). Most T2D children diagnosed during this period were females from minority populations, with a

mean age of onset at around puberty. They were also likely to have a positive family history of the disease, particularly maternal diabetes.

Environmental Risk Factors. As early as 1962, Neel hypothesized that T2D represented a 'thrifty genotype', which had a selective advantage (Neel, 1962). He postulated that in primitive times, individuals who were 'metabolically thrifty' and able to store a high proportion of energy as fat when food was plentiful were more likely to survive times of famine. However, in recent years, most populations experience a continuous supply of calorie-dense processed foods, as well as a decrease in physical activity. This likely explains the rise in T2D prevalence worldwide.

The major environmental risk factors for T2D are obesity ($\geq$ 120% ideal body weight or a body mass index $\geq$ 27 k/m^2) and a sedentary lifestyle (van Dam, 2003; Shaw and Chisholm, 2003). Thus, the tremendous increase in the rates of T2D in recent years has been attributed, primarily, to the dramatic rise in obesity worldwide (Zimmet et al., 2001). It has been estimated that approximately 80% of all new T2D cases are due to obesity (Lean, 2000). This is true for adults and children. In the Pima Indians, 85% of the T2D children were either overweight or obese (Fagot-Campagna et al., 2000). Another study in the US reported that IGT was detected in 25% of obese children age 4-10 years, and in 21% of obese adolescents (Sinha et al., 2002). Undiagnosed T2D was detected in 4% of the adolescents.

In addition to general obesity, the distribution of body fat, estimated by the ratio of waist-to-hip circumference (WHR), also has an impact on T2D risk. WHR is a reflection of abdominal (central)

obesity, which is more strongly associated with T2D than the standard measures of obesity, such as those based on body mass index.

The other major T2D risk factor is physical inactivity. In addition to controlling weight, exercise improves glucose and lipid metabolism, which decreases T2D risk. Physical activity, such as daily walking or cycling for more than 30 minutes, has been shown to significantly reduce the risk of T2D (Hu et al., 2003). Physical activity has also been inversely related to body mass index and IGT. Recently, intervention studies in China (Pan et al., 1997), Finland (Tuomilehto J et al., 2001) and the US (Diabetes Prevention Program Study Group, 2002) have shown that lifestyle interventions targeting diet and exercise decreased the risk of progression from IGT to T2D by approximately 60% . In contrast, oral hypoglycemic medication only reduced the risk of progression by about 30%.

There is also considerable evidence suggesting that the intrauterine environment is an important predictor of T2D risk (Frayling and Hattersley, 2001; Sobngwi et al., 2003), Numerous studies have shown that low birth weight, which is an indicator of fetal malnutrition, is associated with IGT and T2D later in life. However, it is unclear whether low birth weight is causal or related to potential confounding factors that contribute to both poor fetal growth and T2D.

Role of Genetics in the Development of Diabetes

Type 1 Diabetes

First degree relatives have a higher risk of developing T1D than unrelated individuals from the general population (approximately 6% vs. <1%, respectively) (Dorman and Bunker, 2000). These data

suggest that genetic factors are involved with the development of the disease. At present, there is evidence that more than 20 regions of the genome may be involved in genetic susceptibility to T1D. However, none of the candidates identified have a greater influence on T1D risk than that conferred by genes in the HLA region of chromosome 6. This region contains several hundred genes known to be involved in immune response. Those most strongly associated with the disease are the HLA class II genes (i.e., HLA-DR, DQ, DP).

IDDM1. The HLA class II genes, also referred to as *IDDM1*, contribute approximately 40-50% of the heritable risk for T1D (Hirschhorn et al., 2003). When evaluated as haplotypes, DQA1*0501-DQB1*0201 and DQA1*0301-DQB1*0302 are most strongly associated T1D in Caucasian populations. They are in linkage disequilibrium with DRB1*03 and DRB1*04, respectively. Specific DRB1*04 alleles also modify the risk associated with the DQA1*0301-DQB1*0302 haplotype. Other reported high risk haplotypes for T1D include DRB1*07-DQA1*0301-DQB1*0201 among African Americans, DRB1*09-DQA1*0301-DQB1*0303 among Japanese, and DRB1*04-DQA1*0401-DQB1*0302 among Chinese. DRB1*15-DQA1*0602-DQB1*0102 is protective and associated with a reduced risk of T1D in most populations. Recent reports suggest that other genes in the central, class I and extended class I regions may also increase T1D risk independent of HLA class II genes (Nejentsev et al., 1997; Lie et al., 1999).

Individuals with two high risk DRB1-DQA1-DQB1 haplotypes have a significantly higher T1D risk than individuals with no high risk haplotype. The T1D risk among those with only one susceptibility haplotype is also increased, but effect is more modest. Relative risk estimates range from 10 - 45 and 3-7, respectively, for these groups, depending on race (Dorman and Bunker, 2000). In terms of

absolute risk, Caucasian individuals with two susceptibility haplotypes have an approximately 6% chance of developing T1D through age 35 years. However, this figure is substantially lower in populations where T1D is rare (i.e., < 1% among Asians). In addition to *IDDM1*, two other genes are now known to influence T1D risk (Anjos and Polychronakos, 2004). These include *INS* and *CTLA-4*.

Table 1. Several T1D Susceptibility Genes

Gene	Locus	Variant	Estimated RR†
HLA-DQB1	6p21.3	*0201 & *0302	3 - 45
INS	11p15. 5	Class I	1 - 2
CTLA4	2q31-35	Thr17Ala	1 - 2

†RR = relative risk

INS (insulin). The *INS* gene, located on chromosome 11p15.5, has been designated as *IDDM2*. Positive associations have been observed with a non-transcribed variable number of tandem repeat (VNTR) in the 5' flanking region (Bennett et al., 1997; Pugliese et al., 1997) . There are two common variants. The shorter class I variant predisposes to T1D (relative increase: 1 - 2), whereas the longer class III variant appears to be dominantly protective. The biological plausibility of these associations may relate to the expression of insulin mRNA in the thymus. Class III variants appear to generate higher levels of insulin mRNA than class I variants. Such differences could contribute to a better immune tolerance for class III positive individuals by increasing the likelihood of negative selection for autoreactive T-cell clones. The effect of *INS* appears to vary by ethnicity, with lesser effects in non-Caucasian populations (Undlien et al. 1994).

CTLA-4 (cytotoxic T lymphocyte-associated 4). The *CTLA-4* gene is located on chromosome 2q31-35 (Anjos and Polychronakos, 2004), where multiple T1D genes may be located. CTLA-4 variants have been associated with T1D, as well as other autoimmune disease. *CTLA-4* negatively regulates

T-cell function. However, impaired activity, which has been associated with the Thr17Ala variant, may increase T1D risk. Overall, the relative increase in risk for the CTLA-4Ala17 variant has been estimated as ~ 1.5.

Type 2 Diabetes

It has long been known that T2D is, in part, inherited. Family studies have revealed that first degree relatives of individuals with T2D are about 3 times more likely to develop the disease than individuals without a positive family history of the disease (Flores et al., 2003; Hansen 2003; Gloyn 2003). It has also been shown that concordance rates for monozygotic twins, which have ranged from 60-90%, are significantly higher than those for dizygotic twins. Thus, it is clear that T2D has a strong genetic component.

One approach that is used to identify disease susceptibility genes is based on the identification of candidate genes (Barroso et al., 2003; Stumvoll, 2004). Candidate genes are selected because they are thought to be involved in pancreatic β cell function, insulin action / glucose metabolism, or other metabolic conditions that increase T2D risk (e.g., energy intake / expenditure, lipid metabolism). To date, more than 50 candidate genes for T2D have been studied in various populations worldwide. However, results for essentially all candidate genes have been conflicting. Possible explanations for the divergent findings include small sample sizes, differences in T2D susceptibility across ethnic groups, variation in environmental exposures, and gene-environmental interactions. Because of current controversy, this review will focus only on a few of the most promising candidate genes. These include *PPARγ*, *ABCC8*, *KCNJ11*, and *CALPN10*.

Table 2. Several T2D Susceptibility Genes

Gene	Locus	Variant	Estimated RR†
PPARγ	3p25	Pro12Ala	1 - 3
ABCC8	11p15.1	Ser1369Ala	2 - 4
KCNJ11	11p15.1	Glu23Lys	1 - 2
CALPN10	2q37.3	A43G	1 - 4

†RR = relative risk

PPARγ (peroxisome proliferator-activated receptor-γ). This gene has been widely studied because it is important in adipocyte and lipid metabolism. In addition, it is a target for the hypoglycemic drugs known as thiazolidinediones. One form of the *PPARγ* gene (Pro) decreases insulin sensitivity and increases T2D risk by several fold. Perhaps more importantly is that this variant is very common in most populations. Approximately 98% of Europeans carry at least one copy of the Pro allele. Thus, it likely contributes to a considerable proportion (~25%) of T2D that occurs, particularly among Caucasians.

ABCC8 (ATP binding cassette, subfamily C, member 8). This gene encodes the high-affinity sulfonylurea receptor (SUR1) subunit that is coupled to the Kir6.2 subunit (encoded by *KCNJ11*, also known as the potassium channel, inwardly rectifying subfamily J, member 11). Both genes are part of the ATP-sensitive potassium channel, which plays a key role in regulating the release of hormones, such as insulin and glucagon, in the beta cell. Mutations in either gene can affect the potassium channel's activity and insulin secretion, ultimately leading to the development of T2D. Interestingly, *ABCC8* and *KCNJ11* are only 4.5 kb apart, and not far from the *INS* gene. Variant forms of *KCNJ11* (Lys) and *ABCC8* (Ala) genes have been associated with T2D, as well as other diabetes-related traits. Because of the close proximity of these genes, current studies are

evaluating whether they work in concert with each other, or rather have an independent effect on T2D susceptibility.

Since *PPARγ, ABCC8* and *KCNJ11* are the targets of drugs used routinely in the treatment of T2D, there are pharmacogenetic implications for maintaining good glycemic control. Response to hypoglycemic therapy may actually be related one's genotype. Thus, genetic testing may not only help determine who is at high risk for developing T2D, but may also be useful in guiding treatment regimens for T2D.

CAPN10 (calpain 10). *CAPN10* encodes an intracellular calcium-dependent cysteine protease that is ubiquitously expressed (Cox et al., 2004). A haplotype that was initially linked to T2D included an intronic A to G mutation at position 43, which appears to be involved in *CAPN10* transcription. Two amino acid polymorphisms (Thr504Ala and Phe200Thr) have also been associated with T2D risk. However, it has been suggested that the coding and noncoding polymorphisms do not independently influence T2D risk, but instead contribute to an earlier age at diagnosis. Physiological studies suggest that variations in calpain 10 activity effects insulin secretion, and therefore, susceptibility to T2D. Studies from different ethnic groups indicate that the contribution of this locus to increased T2D risk may be much larger in Mexican-American than Caucasian populations.

Maturity-Onset Diabetes of the Young

An uncommon form of T2D (accounting for <5% of all T2D cases) that generally occurs before age 25 years is MODY. MODY is characterized by a slow onset of symptoms, the absence of obesity, no ketosis, and no evidence of beta cell autoimmunity. It is most often managed without the need for

exogenous insulin. MODY displays an autosomal dominant pattern inheritance, generally spanning three generations (Stride and Hattersley, 2002). Because of advances in molecular genetics, it is now known that there are at least six forms of MODY, each of which caused by a mutation in a different gene that is directly involved with beta cell function (Winter, 2003). Table 3 lists the MODY genes that have been identified to date. Because ~15% of MODY patients do not carry mutations in one of these genes, it is anticipated that other genes that cause MODY will be discovered in the near future (Demenais et al., 2003; Frayling et al., 2003; Kim et al., 2004).

Table 3. MODY Genes

Type	Gene	Locus	# Mutations	% MODY
MODY1	*HNF4A*	20q12-q13.1	12	~5%
MODY2	*GCK*	7p15-p13	~200	~15%
MODY3	*HNF1A*	12q24.2	>100	~65%
MODY4	*IPF1*	13q12.1	Few	
MODY5	*HNF1B*	17cen-q21.3	Few	<3%
MODY6	*NEUROD1*	2q32	Few	

GCK (glucokinase). The *GCK* gene is currently the only MODY gene that does not regulate the expression of other genes. Rather, the *GCK* gene plays a key role in glucose metabolism and insulin secretion. Thus, the clinical course of MODY2 patients differs from the prognosis associated with other types of MODY. MODY2 patients have a mild fasting hyperglycemia that is present from birth, and generally stable throughout life. There may be a mild deterioration of normoglycemia with age, but patients with MODY2 mutations are usually asymptomatic. Most are detected during routine medical screening. Women with MODY2 mutations are often diagnosed during pregnancy. However, the outcome of the pregnancy can be influenced by whether the mother and / or fetus carry the mutation. When both mother and fetus are MODY2 positive, there is generally no effect

on birth weight. However, MODY2 negative fetuses are carried by MODY2 positive mothers are typically large for gestational age due to maternal hyperglycemia. In contrast, if the fetus, but not the mother, carries the MODY2 mutation, their birth weight will be reduced by approximately 500g due to reduced fetal insulin secretion, which inhibits growth.

HNF4A (hepatocyte nuclear factor 4-a). Mutations in promoter and coding regions of the *HNF4A* gene cause MODY1. *HNF4A* is expressed in many tissues, including the liver and pancreas. It regulates hepatic gene expression, and influences the expression of other MODY genes such as *HNF1A,* which causes MODY3. In the beta cell of the pancreas, it directly activates insulin gene expression. Mutations in the *HNF4A* gene also have been associated with T2D (Silander et al., 2004).

HNF1A (hepatocyte nucleara factor 1-a). MODY3, the most frequent cause of the disease, results from mutations in the *HNF1A* gene. *HNF1A* is expressed in the liver and pancreas. It can also influence *HNF4A* expression, indicating a connection between MODY1 and MODY3. This suggests that the MODY transcription factors form a regulatory network that maintains glucose homeostasis. In addition to causing MODY3, *HNF1A* mutations have been associated with T1D (Moller et al., 1998; Lehto et al., 1999) and T2D (Pearson et al., 2004).

IPF1 (insulin promoter factor-1). MODY4, which is a rare form of the disease, is due to mutations in the *IPF1* gene. Homozygosity for such mutations has been associated with newborn pancreatic agenesis and neonatal diabetes. Therefore, infants who carry MODY4 mutations tend to be small

for gestational age. Individuals with MODY4 may also develop T2D (Cockburn et al., 2004). *IPF1* regulates expression of glucokinase, insulin and other genes involved in glucose metabolism.

HNF1B (hepatocyte nucleara factor 1-β). MODY5, another rare form of MODY, has also been linked with MODY1 because *HNF1β* regulates *HNF4α*. However, unlike MODY1, MODY5 is also associated with renal cysts, proteinuria and renal failure.

NEUROD1 (neurogenic differentiation factor 1). Mutations in *NEUROD1* are responsible for MODY6. MODY6 is also rare. Together, MODY4, MODY5 and MODY6 comprise less then 3% of all MODY cases. *NERUOD1* is expressed in the beta cells of the pancreas, the intestine and the brain. In the pancreas, it contributes to the regulation of the expression of insulin.

To summarize, all MODY genes are expressed in the islet cells of the pancreas, and play a role in the metabolism of glucose, the regulation of insulin or other genes involved in glucose transport, and/or the development of the fetal pancreas. Because MODY phenotypes vary depending which gene is involved (Table 4), genetic testing may also assist in the treatment of the disease.

Table 4. MODY Phenotypes

Type	Disease Onset	Complications	Treatment
MODY1	Severe	Frequent	Diet, oral agents, insulin
MODY2	Mild	Rare	Diet
MODY3	Severe	Frequent	Diet, oral agents, insulin
MODY4	Moderate	Little data	Oral agents, insulin
MODY5	Severe	Renal cysts	Oral agents, insulin
MODY6	Severe	Little data	Diet, oral agents, insulin

Role of Genetics in the Treatment and Prevention of Diabetes

Type 1 Diabetes

At the present time, there is no way to prevent T1D. Lifelong insulin injections are the only available treatment for the disease. Thus, genetics does not currently play a role in the management or prevention of T1D.

Although a cure for T1D is currently unavailable, several large multi-national investigations have been designed to evaluate a variety of primary and secondary disease interventions (Devendra et al., 2004). The tested interventions have included prophylactic nasal insulin (Diabetes Prediction and Prevention Project (DIPP) in Finland), oral and injected insulin (Diabetes Prevention Trial - 1 (DPT-1) in the US), as well as high doses of nicotinamide (European Nicotinamide Diabetes Intervention Trial - ENDIT), and the avoidance of cow's milk exposure during the first six months of life (Trial to Reduce in Genetically At-Risk (TRIGR) in Finland, US and other countries). These investigations focus on 'prediabetic' individuals identified from families with at least one child with type 1 diabetes. DIPP and TRIGR use HLA-DQB1 screening and recruit only individuals at increased genetic risk. The remaining trials recruit relatives with evidence of beta cell autoimmunity as a pre-clinical marker for disease. To date, none of these interventions have prevented or delayed the onset of T1D (Diabetes Prevention Trial-Type 1 Study Group, 2002; NIDDK, 2003; The ENDIT Group, 2003; Paronen, et al., 2000). However, with the formation of *Type 1 Diabetes TrialNet (www.trialnet.com)*, a collaborative network of clinical centers and experts in diabetes and immunology, new intervention strategies are currently being planned. It is ultimately hoped that through genetic testing, individuals at high risk for T1D could be identified prior to the onset of the disease - at a time when primary prevention strategies could be safely administered. It is most

likely that such predictive genetic testing would be offered to families with an affected individual before it was made available to the general population.

Type 2 Diabetes

Unlike T1D, T2D can generally be prevented by maintaining an age-appropriate body weight and engaging in physical activity. Although public health messages that emphasize a nutritious diet and regular physical activity are now commonplace, they have not been effective in terms of disease prevention. Given the recent obesity epidemic, it is obvious that current intervention strategies are being ignored by a majority of individuals in the general population.

Leaders of the Human Genome Project have predicted that genetic tests will become available for many common disorders during the first decade of the 21st century, permitting persons "to learn their individual susceptibilities and to take steps to reduce those risks" by applying interventions based on "medical surveillance, lifestyle modifications, diet or drug therapy" (Collins and McKusick, 2001). In fact, several companies are now offering genetic susceptibility testing, which can be ordered online by any individual, for conditions such as cardiovascular disease and obesity (Khoury et al., 2004).

Although many scientists and health professionals share this optimistic perspective regarding genetics and disease prevention, others are more pessimistic for a variety of reasons. First, the predictive value of most genetic tests is low (Haga et al., 2003); and risk estimates do not account for well-known environmental determinants of disease. Secondly, it is unclear whether knowledge of one's genetic risk increases motivation to engage in disease interventions. Thirdly, genetic

testing presents educational and information-dissemination challenges that were outlined in detail by the Secretary's Advisory Committee on Genetics, Health and Society (Holtzman and Watson, 1998). These include being able to communicate the validity and utility of proposed genetic tests, as well as the potential risks and benefits of being tested, to individuals who may have little knowledge of human genetics. Fourthly, most health care professionals are currently unqualified to interpret the results of genetic tests; and there are no standards for the use of molecular diagnostics in clinical practice. Fifthly, genetic testing may lead to significant distress, the magnitude of which is likely to vary as a function of actual test results, coping skills and resources, risk perception, optimism, health beliefs and pre-existing depression or anxiety.

These factors directly relate to other concerns such as insurance and employment discrimination, confidentiality and stigmatization based on knowing that one is at high genetic risk. In the near future, genetic testing for T2D and other chronic diseases will most certainly become available. Although it is unclear whether this will actually contribute to the prevention of T2D, it may be beneficial in terms of disease management. Many of the current T2D susceptibility genes of interest are drug targets. Evidence for the role of pharmacogenetics in diabetes is already apparent in treatment approaches for MODY.

Maturity-Onset Diabetes of the Young

The most common causes of MODY are related to mutations in MODY3, MODY2 and MODY1 genes. Although individuals who carry MODY2 mutations have a very mild form of the disease, those who carry MODY1 and MODY3 variants have a much more severe expression that is associated with long-term complications. In addition, there has been a link between MODY3 and MODY5 because of

their interaction in terms of gene expression. However, it is now becoming clear that the metabolic phenotype of individuals with these two forms of MODY is actually quite different (Pearson et al., 2004). To date, little has been known about MODY5 other than its association with renal cysts. However, it now appears that MODY5 is more strongly associated with hyperinsulinemia and dyslipidemia (and more closely related to insulin resistance and T2D) than MODY3. Thus, knowledge about the underlying MODY defect is likely to lead to better management and an improved prognosis for individuals with the disease.

Given the autosomal dominant inheritance of all forms of MODY, individuals with a diabetic parent may also wish to have genetic testing. Early diagnosis MODY may also help reduce the likelihood of long-term complications. In addition, psychological and family adjustments to diabetes may also be improved when the specific form of the disease is known.

Approximately one-third of individuals with MODY3 and MODY1 are each treated by diet, oral agents and insulin. Some individuals with MODY3 have been previously classified as having T1D because of the severity of the disease (Moller et al., 1998; Lehto et al., 1999). It is now known that individuals with MODY3 mutations are extremely sensitive to the hypoglycemic effects of sulfonylureas. Thus, these oral agents are likely to be the treatment of choice of individuals with MODY3. Recently, there have been a number of reports of MODY3 individuals being able to change treatment regimens from insulin injections to oral sulphonylurea agents, with considerable improvement in glycemic control (Shepherd, 2003a; Shepherd and Hattersley, 2004)). This is frequently associated with a positive impact on lifestyle and self image, as well as fear and anxiety about the possibility of stopping insulin. Some individuals, particularly those who have long-term

complications, have become angry because they were previously misdiagnosed and/or treated inappropriately. These reactions have implications for health professionals who need to be knowledgeable about the potential psychological consequences of changing treatment regimens (Shepherd, 2003b).

Future Role of Genetics in Diabetes

Within the next decade, the genes that increase risk of developing all forms of diabetes will likely be known. It is, therefore, important that scientists, health professionals, and members of population at large consider how to maximize the advantages, and minimize the disadvantages of predictive genetic testing for diabetes.

In September 2004, the Office of Genomics and Disease Prevention at the Centers for Disease Control in the US held a meeting entitled "Public Health Assessment of Genetic Tests for Screening and Prevention". One of the objectives of this session was to discuss issues related to the evaluation and utilization of genetic tests. Emphasis was placed on three major barriers: 1) the lack of available population data regarding the contribution of genetic variants to disease susceptibility, 2) the lack of an evidence-based process for the integration of genomics into practice and, 3) the lack of readiness of the health care and public health systems to utilize genetic testing for disease prevention. At the end of the meeting it was apparent that we, as a society, are a long way from the practice of 'genomic medicine'.

With regards to diabetes, addressing the first barrier is most critical at the present time. This barrier pertains to the lack of consistent results across populations with regards to the genetic

determinants of the disease. Failure to replicate study results may be due to a variety of factors, the most important of which may be that different gene-environment interactions operate different populations to increase risk of developing diabetes. Thus, considerably more epidemiologic research will be needed before we know the actual risk associated with particular genetic variants. This also likely means that we will not be able to apply a 'one size fits all' model when it comes to the genetic testing for any of the forms of diabetes.

To fulfill the promise of the Human Genome Project, several issues that warrant careful consideration. First, multidisciplinary teams will be required to translate genetic discoveries from the laboratory to the community. This is, perhaps, best exemplified by the development of new initiatives such as the NIH Roadmap in the US. Scientists will no longer be able to work in isolation, without input of individuals from other professions, if they are to maximize the impact of their research in terms of improving health. In particular, issues such as quality assurance, health risks and benefits, and economics need to be addressed. This will require expertise from persons who have typically worked outside the profession of science. Finally, the ethical, legal and social issues associated with widespread availability and use of predictive genetic tests must been addressed. These include confidentiality, discrimination, diversity, informed consent, keeping up with genetic discoveries and uncertainty. Ideally, consideration of such issues will lead to the development of practice guidelines for diabetes, which will hopefully serve as a model for genetic testing for other complex diseases.

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Genetics and Type 1 Diabetes Online Resources for Diabetes Educators

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PURPOSE

Genetic education Internet sites and peer-reviewed medical literature were reviewed and critiqued to develop tables summarizing online resources for diabetes health professionals.

METHODS

Using Internet search engines, each Web site identified for this project met the following criteria: (1) accurate and valid site content based on widely accepted genetic texts, (2) credibility of the organization that maintained the Web site, (3) ease of navigation, and (4) provision of continuing education credits. PubMed was used to find journal articles using similar criteria.

RESULTS

There were 33 Web sites on genetic education for diabetes health professionals that met the inclusion criteria. The literature search identified 36 articles regarding the importance of genetic education for nurses and other health professionals, as well as information regarding genetics and diabetes.

CONCLUSIONS

Valid and credible information on genetics and type 1 diabetes is available for diabetes health professionals on the Internet and in the medical literature.

Type 1 diabetes is an autoimmune disease that occurs in genetically predisposed individuals. Although the disease typically manifests during childhood and adolescence, it can develop at later ages as well. Several ongoing studies are examining the natural history of diabetes beginning at birth (eg, the Diabetes Autoimmunity Study in the Young [DAISY] in Colorado,[1,2] and the Prospective Assessment in Newborns of Diabetes Autoimmunity [PANDA] in Florida and Georgia[3]). These investigations focus on infants from the general population who screen positive for HLA-DQ alleles that are associated with an increased risk for type 1 diabetes. The Trial to Reduce IDDM in the Genetically At Risk (TRIGR) Study[4,5] is also enrolling infants who are identified by genetic testing of newborns in families where there is already an affected first-degree relative. Parents of babies who have a first-degree relative with type 1 diabetes and who also carry high-risk HLA-DQ alleles are invited to participate in this primary prevention trial that involves a nutritional intervention.

Diabetes educators, nurses, and other health professionals who care for families with type 1 diabetes and/or recruit families for such investigations may be responsible for providing information regarding genetics and/or obtaining informed consent. Because genetic studies can impact study participants, as well as other family members, it is crucial that accurate information be conveyed in a meaningful way. However, many nurses and diabetes educators have never received formal training in human genetics and are unaware of state-of-the-art information regarding genetic testing for type 1 diabetes. Thus, they may not be able to adequately answer questions about the rational for genetic testing or what it means to have a child at high or low risk for type 1 diabetes. Having an understanding of genetic testing is particularly important given that there is currently no medical approach to prevent type 1 diabetes. Although our understanding of the genetics of this multifactorial disorder is incomplete, it is constantly evolving. There is considerable information currently available to nurses and diabetes educators, through informal or continuing education opportunities, that can improve their knowledge about the genetics of type 1 diabetes. Those interested in formal training can also function as genetic counselors for type 1 diabetes and assist families in understanding how type 1 diabetes is

inherited. This information will also facilitate the informed consent process for families who enroll in research studies.

The Conference on Behavioral Science Research in Diabetes,[6] sponsored by the National Institutes of Health (NIH), also emphasized the need to provide accurate risk information; maximize the benefits of determining risk status; minimize distress during risk notification; and educate children, families, and health professionals regarding genetic testing for type 1 diabetes. At the present time, these recommendations are being followed only in a research environment, as proposed by the American Diabetes Association (ADA).[7] However, the NIH-sponsored conference recommendations are also echoed in the final Report of the Task Force on Genetic Testing,[8] which underscored the importance of developing genetics curricula for schools of nursing, public health, and social work; medical schools; and residency training programs. These recommendations directly apply to diabetes educators and nurses who care for families with type 1 diabetes and need access to up-to-date information regarding the genetics of the disease.

Although a plethora of genetic information is available on the World Wide Web (WWW), it can be difficult to find sites that provide accurate and appropriate information for health professionals. For example, a search of the word *genetics* using Internet search engines such as Google (*http://www.google.com*), Yahoo (*http://www.yahoo.com*), and MSN (*http://www.msn.com*) revealed over 6 million sites. Peer-reviewed literature can also provide useful information on genetics for health professionals. PubMed (*http://www.ncbi.nlm.nih.gov/*) is an excellent resource for finding journal articles pertaining to genetics. However, many of these are disease-specific and relate to research findings rather than genetic education.

Thus, we reviewed and critiqued genetic education Internet sites and peer-reviewed literature that would be most appropriate for diabetes educators and nurses working with families with type 1 diabetes in a clinical or research setting. This article provides a summary of the resources that are likely to be of greatest benefit to diabetes healthcare professionals. A companion article on similar Web-based resources for consumers also is published in this issue of *The Diabetes Educator*.

METHODS

Three Internet search engines—Google, MSN, and Yahoo—were used to identify sites using the keywords *health professionals*, *genetics*, *diabetes*, and *education*. Search terms were clustered, such as *professionals and genetics and diabetes and education*, to decrease the number sites found and to provide more focused results. Each Web site identified by the search was ranked according to the following criteria: (1) accurate and valid site content based on textbooks, such as, Thompson and Thompson Genetics in Medicine,[9] as well as a panel of experts in the Department of Human Genetics at the Graduate School of Public Health, University of Pittsburgh, and the Department of Endocrinology at University of Pittsburgh Medical Center; (2) credibility of the organization that maintained the Web site; (3) ease of navigation; and (4) provision of continuing education credits. Sites were excluded if these criteria were not met. The inclusion of criteria were based on guidelines for assessing information quality on the Internet[10] pertaining to issues such as credibility, content, disclosure of purpose of the site, usefulness of links, Web site design ensuring effectiveness of delivery, and interactivity in the form of a route for user feedback (available at: *http://hitiweb.mitretek.org/docs/criteria.html*). Another site provides the Information Quality (IQ) Tool, which assesses the content and layout of the Web site through questions that are answered by the person using the site (available at: *http://hitiweb.mitretek.org/info/index.asp*). To use the IQ Tool, the site address is entered, which opens the page along with a list of questions to answer. The user navigates the selected site to answer the assessment questions. Once completed, the IQ Tool provides a report that contains a review of answers, a score, and information on the importance of what was missing from the site, such as a list of authors and their credentials, and whether their credentials pertain to the topic of the Web site. The score is not a measure of quality but rather indicates how well the Web site provided answers to the IQ Tool questions. The portion of the score that is most informative is the detailed report on what was missing from the Web site; this section tells why the answers to the questions are important. In this way, the IQ Tool provides a way of assessing the quality of a Web site.

A review of the literature on genetic education for health professionals was also conducted, with a special emphasis on articles describing Web-based educa-

tion. The online journal search engine, PubMed (available at: *http://www.ncbi.nlm.nih.gov/*), was used to identify relevant journal articles. The content of each article was reviewed for validity and accuracy of information, as well as for whether it provided useful information for health professionals.

RESULTS

Web-Site Review

The search for Web sites on genetic education for diabetes health professionals identified 33 comprehensive sites that met the inclusion criteria. These Web sites are listed in Table 1, which includes the site address or uniform resource locator (URL), a brief description of the site, and whether the information regarding diabetes or continuing education (CE) credits was provided. Most of the sites had credible sources, valid information, and were easily navigated.

Literature Review

The search of literature published over the last 5 years identified 22 articles regarding the importance of genetic education for nurses (Table 2) and for other health professionals, such as diabetes educators (Table 3). Three articles were identified that provided genetic education information, 2 of which could be used to obtain continuing education credits (Table 4). Eleven articles were identified that discussed how to use the Internet and associated databases, and other resources that may be of use to primary care providers (Table 5).

DISCUSSION

The Human Genome Project recently announced that the sequence of the entire human genome is now complete.[11] This advance will bring about many new discoveries in the field of genetics that diabetes educators and other health professionals will need to access. As the field of genetics expands and the technology and assays available for genetic testing become more reliable, faster, and more easily used, the genetic knowledge that nurses and other healthcare professionals need will only increase. Finding resources for the appropriate level of genetic education can be a challenge. The Internet provides an easy and convenient way to obtain genetics information online for quick reference. However, diabetes health professionals need resources that assist them in obtaining the type of genetic education that is relevant for their needs.

The tables prepared from this extensive search of Web sites and the medical literature are valuable resources for diabetes educators and nurses who want to know about genetics and type 1 diabetes. The goal of this review was to create a relevant and timely resource for diabetes health professionals. This resource is important because it would be impossible for health professionals without formal training in genetics to review and critique existing Internet sites for content, accuracy, etc, and identify those that would best meet their needs. The tables prepared for this review provide a summary of a wide range of excellent genetic information Web sites that will be useful for diabetes health professionals.

The literature search revealed several articles describing how to use and access medical genetics information on the Internet. These articles provide a solid base and an alternative resource for diabetes health professionals who are not particularly computer savvy. A plethora of information regarding the importance of educating health professionals in genetics was also found, which addressed the need for information regarding the genetics of type I diabetes to be readily available and easy to access using the World Wide Web. Although few articles actually provided genetic education, there were many articles describing genetic diabetes research. The research findings on the genetics of type 1 diabetes are extensive but easily accessed through PubMed and other medical journal databases. These journal articles can provide interested diabetes health professionals with more detailed basic science information about the genetics of type 1 diabetes.

Diabetes healthcare professionals who understand basic genetics can also conduct a more thorough assessment of family health, which includes complications of diabetes. The ability to take a detailed family history and to identify potential inherited patterns of disease-associated symptoms is critical. In this capacity, diabetes healthcare professionals have the opportunity to serve as a resource for affected families. In addition to providing genetic information, the purpose of this type of counseling role is to provide families with emotional support and assist them in obtaining the specific genetic counseling that they need. This role is a natural extension of the responsibilities of diabetes educators, who are formally trained in health communication. Thus, they can help families make informed choices about genetic testing, treatment, and potential participation in research, and assist families being better pre-

pared should an at-risk child actually develop type 1 diabetes. Equally important is the role of diabetes health providers in identifying resources that will aid in patient education, which now includes the basic concepts of genetics and genetic risk for type 1 diabetes.

Several of the genetics education Web sites identified for diabetes health professionals included programs for obtaining continuing education credit, which is required to maintain certifications. The School of Nursing at the University of Pittsburgh developed Web-based genetics education modules for oncology nurses that complement an introductory course in human genetics that is taught to second-year nursing students (Y. Conley, XXX Communication, Month 2003). The Web-based version of the course follows the content of the classroom component but also contains specific modules related to cancer genetics. These modules are available to oncology nurses at other institutions, providing an excellent example of the use of the Internet to formally educate nurses and health professionals about the field of genetics. Web-based programs are also easy to update, which is particularly important for a field such as genetics, where new information is being introduced regularly. The development of this program has prompted our group to construct a Web site specifically targeted to diabetes health professionals. The goal of this site, as well as the tables prepared for this manuscript, is to teach diabetes educators and nurses about the genetics of type 1 diabetes for the purposes of increasing their knowledge and helping them become better healthcare providers.

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Table 1.

Web Sites That Provide Genetic Informaiton for Health Professionals

Organization	Web-Site Address (URL)	Description
American Academy of Family Physicians	*http://www.aafp.org/afp/990700ap/core.html*	Provides a list of what a family practice resident should know about medical genetics
American Nurses Association*	*http://www.nursingworld.org/mods/mod170/cegntoc.htm*	Provides CE courses (eg, Genetic Care—An Historical Perspective on Genetic Care) for nurses to learn about the history of genetic care, the role of nurses in genetic care, and the implications of genetic care for nursing practice; CE credits (2 contact hours) available for a fee
American Society of Human Genetics	*http://www.faseb.org/genetics/ashg/educ/002.shtml*	Serves as an educational resource with links to informational sites on genetics
Association of Professors of Human or Medical Genetics: National Coalition for Health Professionals Education in Genetics	*http://www.faseb.org/genetics/aphmg/guidelines.htm*	Provides a list of information that health professionals should know about genetics to better care for patients
Centers for Disease Control and Prevention, Office of Genomics and Disease Prevention Training and Education	*http://www.cde.gov/genomics/training.htm*	Provides a list of online presentations, including slide shows, videos, lectures, and audio clips, geared toward children K-17 years old and health professionals; also includes a list of training opportunities in the field of genetics, a list of core competencies in genetics essential for all healthcare professionals, and a public health workforce genomic competencies list
Center for Genetic Education in Sydney, Austrailia	*http://www.genetics.com.au/Genetics2003/factSheets/34.asp*	Provides a detailed explanation of the genetics of diabetes along with links designed for professionals, students, and patients
Children with Diabetes	*http://www.childrenwithdiabetes.com/d_0n_500.htm*	Provides information about studies being conducted in type 1 diabetes, including links to study Web pages
Cold Spring Harbor Laboratory, Dolan DNA Learning Center, Image Archive on the American Eugenics Movement	*http://www.eugenicsarchive.org/eugenics/*	Provides historic reports, articles, charts, and pedigrees considered scientific fact in their day; also includes recently written historic articles detailing the events leading up to legislation condoning eugenic practices and the events after this legislation led to banning of eugenic practices
Columbus Networks	*http://dirs.educationworld.net/cat/5860705*	Provides links to Web sites of professional organizations
Freising-Weihenstephen	*http://www.weihenstephan.de/~schlind//genglos.html*	Serves as a hypermedia glossary of genetic terms; the number of terms is relatively short but the site can be useful for a quick search for basic genetics terminology
Genetic Education Materials Database	*http://www.gemdatabase.org/GEMDatabase/index.asp*	Provides a list of public health genetics policy documents and clinical genetics educational materials; these documents and materials are listed as hyperlinks that route the user to pages that will say if the material is available; some of the material is not free; a detailed search of the database can be performed to find specific sites of interest
Genetics Education and Counseling Program University of Pittsburgh	*http://www.pitt.edu/AFShome/e/d/edugene/public/html/resource/index2.html*	Provides many links to sites of interest to genetic counselors, medical geneticists, and clinical geneticists

URL=uniform resource locator.
**Cointinuing education (CD) credits available.*

Table 1.

Web Sites That Provide Genetic Informaiton for Health Professionals (continued)

Organization	Web-Site Address (URL)	Description
Genome News Network	*http://www.genomenewsnetwork.org/whats_a_genome/Chp1_1_1.shtml*	Provides an online book (*What's a Genome?*) about genetics in a simple, easy-to-understand format
GlaxoSmithKline: Genetic	*http://genetics.gsk.com/edu.htm*	Provides links to professional meetings, courses, workshops, and conferences; not much content for genetic education
International Society of Nurses in Genetics	*http://www.globalreferrals.com/isong.html*	Provides information specifically for nurses working in the field of genetics; mission is to foster scientific, professional, and personal development of members in the management of genetic information
Johns Hopkins University, Online Mendelian Inheritance in Man (OMIM)	*http://www.ncbi.nlm.nih.gov/omim/*	Serves as a database for information on genetic conditions; excellent resource for text, graphics, and pertinent links for almost all known human genetic disorders
Joslin Diabetes Center*	*http://www.joslin.harvard.edu/main.shtml*	Provides an overview of diabete, information on managing diabetes, professional education, research, Joslin's local and national services, employment opportunities, and fundraising
March of Dimes*	*http://www.marchofdimes.com/professionals/682.asp*	Provides online education modules, lecture series, and online curricula in genetics for physicians; opportunities exist for continuing education credits for professionals and researchers but can be difficult to find
National Coalition for Health Professional Education in Genetics*	*http://www.nchpep.org/*	Provides presentations from annual meetings, educational resources (some CDs), core competencies in genetics for healthcare providers, and other useful resources
National Human Genome Research Institute	*http://www.genome.gov/*	Provides information for teachers of grades K-12 as well as professional training and career development
National Institutes of Health	*http://www.search.info.nih.gov/grow/*	Serves as a search engine that provides a ranked listing of useful Web resources related to the search terms
National Institutes of Health Office of Rare Diseases	*http://rarediseases.info.nih.gov/*	Provides links to resources pertaining to over 7000 rare diseases
National Newborn Screening and Genetics Resource Center	*http://genes-r-us.uthscsa.edu/resources/genetics/primary_care.htm*	Provides materials (PDF documents) for training primary care workers in geetics
National Society of Genetic Counselors	*http://www.nsgc.org*	Provides information about genetic counseling and sources of genetic education
Oncology Nursing Society	*http://www.ons.org/xp6/ONS/Clinical.xml/GeneticsToolkit.xml*	Provides a genetics in cancer tool kit for educating nurses in the genetics of cancer
Overlake Medical Library	*http://www.overlakehospital.org/medical.htm#Genetics*	Provides links to other informational sites
Type 1 Diabetes Genetics Consortium	*http://www.t1dgc.org/*	Serves as a resource for organizing international efforts to identify genes that determine an individual's risk of type 1 diabetes

URL=uniform resource locator.
**Cointinuing education (CD) credits available.*

Table 1.

Web Sites That Provide Genetic Informaiton for Health Professionals (continued)

Organization	Web-Site Address (URL)	Description
U.S. Department of Energy	*http://public.ornl.gov/hgmis/external/category.cfm?category=Education*	Provides links to a variety of topics on genetics
University of Iowa Health Care: Virtual Hospital	*http://www.vh.org/*	Serves as a search engine to find informational documents within the site
University of Iowa Virtual Children's Hospital	*http://www.vh.org/Providers/Textbooks/ClinicalGenetics/Contents.html*	Provides information on clinical genetics in a textbook style; Clinical Genetics: A Self-Study for Health Care Providers—includes 4 lessons: Lesson 1, Genetic Concepts and Genetic Tests; Lesson 2, Assessment Strategies—How to Identify Families Who Might Benefit from Genetic Services; Lesson 3, Genetic Services—What Are They:; Lesson 4, Making a Referral; and 5 appendices: Reasons for Referral; Glossary of Terms and Abbreviations; Annotated Bibliography of Resources; Educational Tools and Resources; and State Services
University of Kansas Medical Center	*http://www.kumc.edu/gec/geneinfo.html*	Provides information for genetics professionals, including clinical, research, and educational resources for genetic counselors, clinical geneticists, and medical geneticists
University of Kansas Medical Center	*http://www.kumc.edu/gec/prof/soclist.html*	Provides a list of professional societies and links to their Web sits
University of Washington School of Medicine, Gene Clinics	*http://www.geneclinics.org*	Provides reviews about genetic conditions and laboratories that do genetic testing; excellent source of information for clinical health professionals

URL=uniform resource locator.
**Cointinuing education (CD) credits available.*

Table 2.

Literature Pertaining to the Importance of Genetic Education for Nurses

Citation	Description
Burton and Stewart. FromMendel to the Human Genome Project: the implications for nurse education. Nurse Educ Today. 2003;23:380-387.	Describes how scientific discovery will impact nursing education; covers topics such as classical and molecular genetics, the Human Genome Project, and how its discoveries will change nursing practice
Jenkins, Dimond. Preparing for the future through genetics nursing education. J Nurs Scholarsh. 2001;33:191-195.	Presents important factors for the education of nurses in genetics through a survey of 356 nurses, 228 identified as experts in genetics and 128 identified as potential users of genetic education; 398 items from this survey were identified as potential consequences of genetic education; provide a template for genetic education programs for nurses
Jenkins, Prows. Recommendations for educating nurses in genetics. J Prof Nurs. 2001;17:283-290.	Describes the importance of nurses having an education in genetics and recommends what information nurses should be taught
Kenner. National Coalition for Health Professional Education in Genetics. AACN Clin Issues. 1998;9:582-587.	Describes the goals of the National Coalition for Health Professional Education in Genetics (NCHPEG); provides an overview of the relationship of NCHPEG to acute and critical care nurses.
Lashley. Genetics in nursing education. Nurs Clin North Am. 2000;35:795-805.	Describes the importance of genetic education for nurses, including genetic roles and necessary genetic knowledge for nurses
Lea, Anderson. A multiplicity of roles for genetic nursing: building toward holistic practice. Holist Nurs Pract. 1998;12: 77-87.	Identifies the roles of nurses in genetic nursing
Lea, Feetham. Genomic-based health care in nursing: a bidirectional approach to bringin genetics into nursing's body of knowledge. J Prof Nurs. 2002;18:120-129.	Explores how nursing hs integrated human and clinical genetics into practice and scholarship; includes a review of nursing education literature and a survey of 15 genetics nursing and nursing leaders on such topics as key national initiatives, genetics research training programs, and genetics education models; presents recommendations on how to incorporate genetic education that builds on previous work into nursing education programs
Monsen, Anderson. Nursing education and genetics. Miles to go before we sleep. Nurs Health Care Perspect. 2000;21:34-37.	Describes a study in which 42 state boards of nursing (SBNs) asking the questions "What are the SBN requirements for genetic content in basic nursing education programs?" and "Is genetics included in nursing course textbooks?" presents conclusions that gentics is covered in nursing curricula but is not recognized as an important area of focus, meaning that clinicians, educators, and researchers have limited exposure to genetics
Terry, Cedar. An overview of education and 'new genetics'. Nurs Stand. 2001;15:38-40.	Discusses the importance of educating nurses about genetic developments for the purpose of applying the knowledge to practice; provides useful Internet sites as resources

Table 3.

Literature Pertaining to the Importance of Genetic Education for Health Professionasl

Citation	Description
Bell. The new genetics in clinical practice. BMJ. 1998;316:618-620.	Reviews the ways in which genetic information impacts clinical practice
Burke, Emery. Genetics education for primary-care providers. Nat Rev Genet. 2002;3:561-566.	Describes the need for genetics education of primary care providers and current efforts in genetics education in the United States and the United Kingdom; also provides recommendations regarding how to educate primary care providers in genetics
Burton, Shuttleworth. Genetics education for midwives. Midwives (Lond). 2003;6: 162-164.	Describes a strategy to meet the educational needs of midwives and other health professionals being asked to take on new roles in genetics
Collins, Bochm. Avoiding casualties in the genetic revolution: the urgent need to educate physicians about genetics. Acad Med. 1999;74:48-49.	Discusses how the Human Genome Project will impact health care and how healthcare providers will need to be educated to provide the best possible patient care
Collins. Preparing health professionals for the genetic revolution. JAMA. 1997;278: 1285-1286.	Discusses the implications of having healthcare providers who are competent in genetics; emphasizes that there will not be enough genetic counselors and medical geneticists in the future to meet the demands for testing and services
Fetters, Doukas. Family physicians' perspectives on genetics and the human genome project. Clin Genet. 1999;56: 28-34.	Describes a study to determine family physicans' attitudes and beliefs about human genetics research and the Human Genome Project; results show that study participants did not feel that genetics has a significant impact on their practics but major clinical changes are expected in the future; many study participants felt there have been inadequate educational opportunities to learn about geneticcs
Fineman. Qualifications of public health geneticists? Community Genet. 1999;2: 113-114.	Describes the knowledge base, skills, and attitudes needed to be a public health geneticist
Greendale, Pyeritz. Empowering primary care health professionals in medical genetics: how soon? How fast? How far? Am J Med Genet. 2001;106:223-232.	Discusses the importance of medical genetics in clinical outcomes and the likelihood that positive attributes of medical genetics will be preserved in a primary care model of medical genetics service delivery
Kinmonth, Reinhard. The new genetics. Implications for clinical services in Britain and the United States. BMJ. 1998;316:767-770.	Describes the importance of having primary care teams with skills to assess genetic risk of disease; discusses the implications of gene testing and control access to specialist services; presents guidelines for referral to consultation and counseling; summarizes how to integrate genetic risk assessment into medical practice
Kolb, Aguilar. Genetics education for primary care providers in community health settings. J Community Health. 1999;24:45-59.	Discusses the effects of a genetics education program on the knowledge and attitudes of primary care providers in community health settings; explains how subjects had inadequate knowledge before an education program was completed and a significant increase in knowledge after the education program was completed; presents authors' conclusions that primary care providers can learn complex materials and new skills to assist their patients in a short period of time

Table 3.

Literature Pertaining to the Importance of Genetic Education for Health Professionasl (continued)

Citation	Description
Lapham, Kozma. The gap between practice and genetics education of health professionals: HuGEM survey results. Genet Med. 2000;2:226-231.	Describes how this study was done to determine the genetic education needs of dietitians, occupational therapists, physical therapists, psychologists, speech-language specialists, and social workers; presents results showing that most study participants worked with clients with genetic conditions but lacked the knowledge and skills needed to deal with these clients
Mountcastle-Shah, Holtzman. Primary care physicians' perceptions of barriers to genetic testing and their willingness to participate in research. Am J Med Genet. 2000;94:409-416.	Describes the barriers and motivations to appropriate diffusion of genetic services into primary care practice and the willingness of primary care providers (PCPs) to participate in clinical studies to assess the safety and effectiveness of emerging genetic technolgies (study involved interviews of 60 PCPs and written surveys of 157 PCPs); presents authors' conclusions that most physicians do not see genetics as important in their practice but anticipate greater need in the future; also discusses sufficient physician interest in participation in research to allow large-scale, collaborative, practice-based evaluation
Recommendations of core competencies in genetics essential for all health professionals. Genet Med. 2001;3: 155-159.	Presents the committee report of the Core Competency Working Group of the National Coalition for Health Professional Education in Genetics, which describes the genetics knowledge, skills, and attitudes needed by all health professionals to provide effective health care to patients

Table 4.

Literature Pertaining to Providing Genetic Education

Citation	Description
Lea. What nurses need to know about genetics. Dimens Crit Care Nurs. 2002;21:50-60; quiz 60-51.*	Addresses basic genetics, mitosis and meiosis, mutations, inheritance patterns, multifactorial conditions, chromosomal abnormalities, genetic assessment, ethics, and indications for making a referral; quesitons at the end of the article can be submitted for continuing education credits
Monsen, Anderson. Continuing education for nurses that incorporates genetics. J Contin Educ Nurs. 1999;30:20-24; quiz 46-27.*	Outlines and highlights the importance of incorporating new genetic advances into clinical practice, research, and education; describes a survey of 43 nursing specialty organizations to determine educational offerings in genetics and presents conclusions that alert leaders in continuing education about the need for education in genetics
Pinsky, Pagon. Genetics through a primary care lens. West J Med. 2001;175:47-50.	Describes why genetics is important for primary care physicians through use of case examples; teaches how to use the family history in risk assessment with pedigrees and how to evaluate the genetic information that is important for risk assessment

**Continuing education (CE) credits available.*

Table 5.

Literature Providing Information on Use of the Internet and Sites Available for Primary Care Providers

Citation	Description
Hetteberg, Trangenstein. Genetics and the World Wide Web: an introduction. Neonatal Networks. 1999;18:9-13.	Presents an overview of the World Wide Web, offers examples of genetics-related Web sites, and illustrates their applicability to neonatal nursing
Osborn, Lee. Resources for human genetics on the World Wide Web. Mol Med Today. 1997;3:370-373.	Describes Internet resources for the human Genome Project, education, and human genetic diseases, and sites that the authors found most informative and original
Ouellette. Internet resources for the clinical geneticist. Clin Genet. 1999;56:179-185.	Reviews resources (relevant to the clinical geneticist) from the National Center for Biotechnology Information at the National Library of Medicine at the National Institutes of Health
Pagon, Pinsky. Online medical genetics resources: a US perspective. BMJ. 2001;322:1035-1037.	Describes Web sites for genetics professionals, primary care providers, and patients, including what the authors felt was missing; focuses on health professionals in the United States
Peters, Sikorski. Navigating to knowledge. Tools for finding information on the Internet. JAMA. 1997;277:505-506.	Describes how to find information on the Internet, covering such topics as finding Web pages, newsgroup postings, and people
Sikorski, Peters. Genomic medicine. Internet resources for medical genetics. JAMA. 1997;278:1212-1213.	Describes specific Web sites, many of which are useful for health professionals as well as for patients and the general public
Sikorski, Peters. Internet anatomy 101. Accessing information on the World Wide Web. JAMA. 1997;277:171-172.	Describes the Internet and how to use it in a simple, straightforward manner; covers basic terminology such as HTTP and URL and what the Internet is
Sikorski, Peters. Medical literature made easy. Querying databases on the Internet. JAMA. 1997;277:959-960.	Describes how to use databases and extract the desired information from these databases; is helpful for those trying to do a literature search
Stewart, Haites. Online medical genetics resources: a UK perspective. BMJ. 2001;322:1037-1039.	Describes sites for genetics professionals; sites for doctors and other primary care professionals; sites for patients, families, and caregivers; sites for public health professionals and policymakers; and genetics newsletters, with a focus on health professionals in the United Kingdom
Trangenstein, Hetteberg. Genetics on the World Wide Web. AACN Clin Issues. 1998;9:574-581.	Describes a variety useful of genetics-related Web sites for health professionals interested in genetics; explains how authors selected sites and searched the Web
van Steensel, Winter. Internet databases for clinical geneticists—an overview. Clin Genet. 1998;53:323-330.	Summarizes databases that are important to clinical geneticists, including databases for the procurement of dysmorphologic information and diagnosis; also describes how to find research information on the Web, keep up with recent developments, and use search engines to find information

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Genetics and Type 1 Diabetes: Online Resources for Patients

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PURPOSE

This Web-based review was undertaken to compile online resources for diabetes educators on genetics and, specifically, the genetics of type 1 diabetes that would provide helpful and accurate information for the public.

METHODS

Keyword searches were performed to identify Web sites for genetics education for the lay public and those specifically geared toward children/young adults. Web sites were critiqued based on credibility (source, currency, relevance/utility), content (accuracy), and design (accessibility, logical organization). Additional keyword searches were conducted to find sites describing the genetics of type 1 diabetes, which were evaluated for content validity.

RESULTS

The Web sites selected for general genetics education contain accessible, credible, and accurate information about basic genetics in an easy-to-follow format with both text and visual aides. Although these sites adequately educate the public about genetics, only diabetes-specific Web sites discussed the relationship between genetics and risk for type 1 diabetes associated with high-risk HLA alleles.

CONCLUSIONS

In this genomic age, providing genetics information is an important consideration for healthcare professionals. Educational tools that specifically address the genetics of type 1 diabetes are urgently needed to fill the current information gaps on the Internet.

the vast amount of information provided by the Human Genome Project has accelerated efforts to elucidate the hereditary components of complex diseases, including type 1 diabetes mellitus. These endeavors are highly anticipated based on their potential to revolutionize standard approaches to the diagnosis, treatment, and prevention of common diseases. In particular, there is substantial evidence that specific genes located in the HLA region of chromosome 6, as well as in other regions of the human genome, increase susceptibility for type 1 diabetes.[1-4]

Recent developments in molecular genotyping for high-risk HLA-DQ alleles have already allowed researchers to identify individuals with a genetic predisposition for type 1 diabetes. Several ongoing natural history studies and clinical trials for type 1 diabetes are using the information provided by molecular testing to recruit participants who are genetically at risk. For example, the Diabetes Autoimmunity Study in the Young (DAISY) in Colorado and the Prospective Assessment in Newborns for Diabetes Autoimmunity (PANDA) in Florida and Georgia are 2 current prospective natural history studies investigating the genetic and environmental precipitants of autoimmunity associated with type 1 diabetes.[5-7] The focus of both of these research initiatives is to identify newborns and children with an increased susceptibility to type 1 diabetes by screening for high-risk HLA alleles. Through ascertainment and follow-up of those with a high genetic propensity for type 1 diabetes, researchers hope to determine precisely how the complex interrelationships among genes, environmental agents, and the immune system work to promote disease progression.

Another investigation based on newborn genetic screening is the Trial to Reduce Insulin-Dependent Diabetes in the Genetically at Risk (TRIGR).[8,9] This multinational primary prevention trial is examining whether delayed exposure to intact food proteins contained in cow's milk formula reduces the likelihood of developing type 1 diabetes among genetically predisposed infants. Infants are eligible if they have a first-degree relative with type 1 diabetes and also have inherited high-risk HLA alleles. The intervention group

of mothers receives a hydrolyzed trial formula lacking intact cow's milk protein while the control group receives a nonhydrolyzed cow's milk formula.

The need for accurate information about genetics and type 1 diabetes is rapidly increasing as individuals with type 1 diabetes become more aware of the use of genetic testing in research studies and as the lay public learns about genes that confer high disease risk. Accordingly, diabetes educators will become a primary resource for genetic information for patients who are eager to learn more about the putative link between genetic factors and risk for the disease. The Internet provides a unique means of accessing and distributing educational materials about the basic genetics of diabetes to a large and diverse audience. This online resource can therefore serve as a valuable gateway for diabetes educators to introduce genetics concepts and new genetic discoveries to their patients.

Although numerous online resources for genetics education are geared toward the general public, there is a relative paucity of information on the Internet dedicated to the genetics of complex diseases such as type 1 diabetes. In addition, the lay community may not be able to assess the quality and accuracy of resources for genetics education that they find through Web-based searches. Thus, the purpose of this review is to highlight helpful and valid information for the lay public regarding the existing Internet resources for genetics education as well as those that describe the genetics of type 1 diabetes. To our knowledge, this review is the only available resource of this kind. A companion article in this issue of *The Diabetes Educator*, "Genetics and Type 1 Diabetes: Online Resources for Diabetes Educators," summarizes similar Web-based resources for diabetes educators interested in the genetics of type 1 diabetes.

METHODS

To locate the currently available Internet resources for genetics education, extensive keyword searches were performed. The keyword phrases *genetic education* and *genetic education for the public* were entered into popular search engines, including Yahoo (*http://www.yahoo.com*) and Google (*http://www.google.com*). Additional sites were located by browsing the links for genetics information that were included in the resulting

Web-sites. Sites that were geared specifically toward genetics education for children/young adults were also identified through these comprehensive searches.

The main Web sites elicited by these keyword searches were then evaluated according to criteria suggested by the Mitretek Systems, Health Information Technology Institute (HITI), which relate to the quality of Web-based health information.[10] For the purpose of this article, Web site assessment primarily focused on credibility, content, and design, as outlined by HITI. Web site credibility was evaluated by taking into account the source, currency, and relevance/utility of the information. Sites that did not display the authority responsible for posting the information were excluded. Currency was ascertained through any dates listed on the Web site (eg, copyright date and date of content posting) but was not used as an exclusion criteria. As long as the sites discussed basic genetic concepts, they were considered relevant and useful to the perspective audience. Content assessment of the identified Web resources involved a review of the accuracy of the information presented. If the explanations of basic genetic terminology on a Web site were consistent with the factual information from general medical genetics textbooks, such as Thompson and Thompson,[11] the site was considered to have content validity, as determined by a panel of experts at the University of Pittsburgh. The accessibility and logical organization (navigability) of the general genetics sites served as the evaluation criteria for Web-site design. Sites that could be located by current Web browser technology were classified as accessible and, therefore, eligible for inclusion. Web-based resources considered to be logically organized presented basic genetics in an easy-to-follow format using a balance of text and either graphics, animation, or interactive components to maintain interest and enhance learning. In contrast, sites that did not incorporate visual aides to supplement text and facilitate comprehension were excluded, as well as sites with only technical explanations of genetics that were not appropriate for the lay public.

Internet resources that described the genetic components of type 1 diabetes were located by exploring general diabetes Web sites and by performing keyword searches for the phrase *genetics and type 1 diabetes* in several popular search engines, including Yahoo and Google. Criteria for selecting these sites were based strictly on content, or whether they present-

ed valid information about the association between genetics and risk for type 1 diabetes that was targeted to the lay public. Web sites that did not specifically discuss the HLA susceptibility genes for type 1 diabetes were excluded. Because information on the Web pertaining to the genetics of type 1 diabetes is virtually nonexistent, the selection criteria for these sites were less stringent compared with the criteria for the general genetics sites.

RESULTS

The Internet sites considered to most effectively educate users about general genetic concepts are described in Table 1. Web sites that provided genetics education for children/young adults are displayed in Table 2. All of the sites included in Tables 1 and 2 indicate an authority and communicate relevant, useful, and accurate information about basic genetics concepts within an educational framework. In general, the selected sites are up-to-date. Regarding Web site design, all of the selected sites consisted of text along with visual components, although many also had unique features such as glossaries and interactive modules.

Few sites, however, explained the concept of genetic risk for complex diseases, the interaction between genes and the environment as causal factors for multifactorial diseases, or the ethical and psychological issues associated with genetic testing and disclosure of personal genetic information. Moreover, only one of these Web sites discussed genetics in relation to type 1 diabetes, which was presented in the form of a fact sheet. Although the existing Web-based resources for genetics education satisfy the need for genetic knowledge among the lay public, at-risk families, and individuals with type 1 diabetes, overall, they do not address the link between genetic factors and disease susceptibility. Likewise, many general diabetes Web sites are easily accessed yet only briefly mention the genetic basis of type 1 diabetes without further elaboration. The select few Web sites that provide a more thorough coverage of the relationship between genetics and risk for type 1 diabetes associated with high-risk HLA alleles are presented in Table 3.

CONCLUSIONS

The tables developed for this review represent valuable educational resources for patients with type 1 diabetes, their family members, and the general public. These tasks can easily be made available in clinic waiting

rooms or distributed as part of the informed consent process for a research study. This information is pertinent to all sectors of the population, particularly given the evidence that the incidence of type 1 diabetes is increasing among children and the paucity of information targeted toward the lay public on the genetics of type 1 diabetes. Because some of the sites also addressed the environmental risk factors for the disease, they may be especially important to parents who may blame themselves when their child is diagnosed with type 1 diabetes. Therefore, this review addresses a major gap that diabetes educators are increasingly being asked to fill.

The Internet can serve as an instrumental modality for transmitting genetics education programs on a population-wide scale. The benefits of Web-based genetics education for type 1 diabetes are multidimensional. For example, a fundamental knowledge of genetics in relation to type 1 diabetes would help the lay public in several ways: (1) to comprehend genetic susceptibility, and the putative genetic and environmental factors that influence disease development; (2) to interpret the findings of new research investigations; (3) to assess the benefits and limitations of genetic testing; and (4) to consider the ethical, legal, and psychosocial issues raised by genetic advances for type 1 diabetes.

As prominent discoveries in the genetics of type 1 diabetes are used in research and applied to standard medical and public health practice, providing genetic information becomes a paramount consideration for all healthcare professionals, especially diabetes educators. In this genomic era, it is necessary for the lay community to have access to educational programs that describe the genetics of type 1 diabetes. This critical need exists because researchers in several states are already screening newborns and children from the general population for HLA alleles associated with high disease risk. In particular, the public needs to be knowledgeable about basic genetic concepts, including genes, DNA, and multifactorial inheritance. The tables prepared for this review meet this important need.

With regard to the patient population and those at-risk for type 1 diabetes, diabetes educators could use Internet-based resources to promote a more comprehensive understanding of the disease. The diabetes educators could share this list of online resources with their patients to help them locate credible and meaningful genetic information. Even patients without

home computers can conveniently access the information online at public libraries. When children are initially diagnosed with type 1 diabetes or identified as having a high genetic risk, their parents are often interested in finding all of the available information. At the same time, they may be approached by researchers eager to recruit study participants. By fostering genetic literacy about type 1 diabetes, online educational tools devoted to the genetics of type 1 diabetes can thereby function as decision aides for the informed consent process for genetic testing and research participation. However, the genetic information available online is definitely not a substitute for a genetic counselor. These specially trained health professionals can take a detailed family history, provide accurate risk assessment, thoroughly explain the risks and benefits of genetic testing, and offer psychosocial support to at-risk families to promote informed decision making.

Beyond the scope of their benefit to participants in research studies, online educational tools that explain the genetics of type 1 diabetes encourage the public to become informed healthcare consumers. Tables I and 2 can be incorporated into standard health care and made widely accessible to the lay public. Additional advantages of Web-based approaches to genetics education include the ability to convey information in an expedient manner, cost-effectiveness, ease of updating and modifying informational content as new discoveries occur in the dynamic field of medical genetics, and opportunities for self-paced learning and immediate user feedback.

From a broader perspective, the virtual explosion in the amount of genetic information accessible on the Web has significant implications for public health policy related to regulating the quality of this information. As the number of genetics sites on the Internet for the general public continues to increase, healthcare professionals must assume the responsibility for assessing the content of these new resources, on behalf of their patients, to ensure their credibility and accuracy. Evaluating these sites by standard criteria, such as the HITI guidelines, will become imperative for healthcare professionals who use these resources to educate their patients. Therefore, this review addresses a growing need. A major challenge confronting healthcare professionals in the current genomic age is encouraging the lay public to become more knowledgeable about the genetics of common diseases, such as type 1 diabetes. Educa-

tional initiatives that address the multifaceted informational needs of patients, their families, and the lay public should help these individuals understand how genetics fits into the overall picture of type 1 diabetes. The development of novel Web-based tutorials for the genetics of type 1 diabetes will hopefully provide a model for creating, implementing, and evaluating future online resources aimed at increasing awareness of the genetic and environmental determinants of risk for other complex diseases. This review and the resources it has compiled are a first attempt to address these issues by directing patients and the general public to Web sites that provide both accurate and appropriate genetic information.

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Table 1.

Genetics Education Sits for the Public

Site/URL	Authority	Special Features	Comments
Access Excellence Resource Center—Understanding Gen Testing *http://www.accessexcellence.org/AE/AEPC/NIH/index.html*	US Dept of Health and Human Services, Public Health Service, National Institutes of Health, National Cancer Institute	Covers basic genetics with a focus on genetic testing and its associated risks and benefits; includes glossary	Not interactive; mainly text based with several graphics, indepth coverage of genetic testing
Blazing a Genetic Trail *http://www.hhmi.org/genetictrail/*	Howard Hughes Medical Institute	Series of professionally written articles on human genetics concepts; includes glossary; Spanish version available	Not interactive; detailed text based examination of genetic concepts with little focus on the basics
Cracking the Code of Life *http://www.pbs.org/wgbh/nova/genome/*	Nova Online	Online viewing of the Nova program, "Cracking the Code of Life" (4/01); includes interviews/articles about genetic topics, interactive modules, glossary, and educational resources	Online video viewing is a great idea; nice interactive components; minimal information about basic genetics
DNA: Heredity and Beyond *http://library.thinkquest.org/20830/main.htm?tqskip1-1*	Created for the 1998 ThinkQuest Competition by student Barret Parker and Matthew Heard	Textbook format covering basic genetics with sections about genetic technologies and ethics; includes glossary, interactive forum to exchange opinions	Mainly text based with some graphics; good information about new genetic technologies
Dolan DNA Learning Center *http://www.dnalc.org/*	Cold Spring Harbor Laboratory	Online textbook, *DNA From the Beginning*; includes animation, image gallery, video interviews, problems, and links for many basic genetic concepts; provides genetic disease information	Comprehensive detailed examination of genetics supplemented with great interactive tools
Gene CRC Learning Centre *http://www.genecrc.org/site/lc/index_lc.htm*	The Cooperative Research Centre for Discovery of Genes for Common Huma Diseases	Printable fact pages with a more detailed look at specific genetic topics; includes glossary, educational resources	Not interactive but easy-to-follow simple overview of basic genetics
Gene Stories . . . The Basics of Being *http://www.bbc.co.uk/health/genes/*	BBCi	Indepth look at basic genetics and related topics with some interactive features; includes information about genetic disorders, genetic testing, ethical issues, and the role of genes in development	Wonderful variety of genetic information presented in a nontechnical manner; includes several outstanding interactive components
Genetic Science Learning Center *http://gsle.genetics.utah.edu/*	Eccles Institute of Human Genetics at the University of Utah	Flash interactive modules covering basic genetics; includes genetic disease corner, sections on stem cell research and cloning; Spanish version available	Great graphics; easy to navigate; Flash modules can take awhile to load; material is simple and easy to follow

URL=uniform resource locator.

Table 1.

Genetics Education Sits for the Public (continued)

Site/URL	Authority	Special Features	Comments
Genetics Education Program *http://www.genetics.com.au/*	Centre for Genetics Education: New South Wales Dept of Health (Sydney, Australia)	Printable PDF fact sheets about basic genetics, genetic disorders, inheritance patterns, genetic technologies; includes a guide to drawing a family health gree, glossary	Not interactive; good quality basic information on genetic concepts; text supported with simple graphics
Genetics @ GlaxoSmithKline *http://genetics.gsk.com/generalpublic-flash.htm*	GlaxoSmithKline	Glash interactive modules explaining basic genetics; includes glossary, self-test of knowledge, FAQs, and teacher resources	Modules capture user interest through great use of graphics and voiceovers, excellent animations, easy-to-understand information
Genomic News Network *http://www.genomenewsnetwork.org/whats_a_genome/Chp1_1_1.shtml*	Center for the Advancement of Genomics	Online book with explanations of basic genetic concepts from a genomics perspective	Not interactive; includes text-rich format supported with some graphics
Genomics and Its Impact on Science and Society: A 2003 Primer *http://www.ornl.gov/TechResources/Human_Genome/publicat/primer2001/index.html*	US Dept. of Energy, Human Genome Program	Online publication with discussions of basic genetics, the Human Genome Project, and the benefits, societal concerns, and future of new genetics; includes genetic dictionary	Not interactive; text complemented by graphical displays, only a brief description of basic genetic terms
The Human Genome: Your Genes, Your Health, Your Future *http://www.wellcome.ac.uk/en/genome/index.html*	The WellcomeTrust	Exploration of genetics, the human genome, genetic technologies, and the impact of genes on health, disease, and society; includes Flash interactive modules, glossary	Thorough review of genetics and genomics; includes current genetic news updates and high quality interactive features
A Revolution in Progress: Human Genetics and Medical Research *http://history.nih.gov/exhibits/genetics/*	National Institutes of Health, Online Exhibit of the DeWitt Stetten, Jr, Museum of Medical Research	Introduction to genetics with details about diagnosis/treatment of selected genetic disorders, use of genetics in medicine, Human Genome Project, and ethical topics; includes glossary	Not interactive except for one embedded question; nice mix of text and great graphics to facilitate learning without losing user interest
The Tech—DNA: The Instruction Manual for All Life *http://thetech.org/exhibits_events/online/genome/overview.html*	The Tech Museum of Innovation	Interactive look at information about DNA and chromosomes; presents different ethical scenarious that place you in the position of a parent, doctor, judge, patient, and voter	Text supplemented with nice interactive images but no mention of genes
Your genome.org *http://www.yourgenome.org/*	Wellcome Trust Sanger Institute	Genetic information presented at beginner, intermediate, and advanced levels; includes FAQs, news, and discussion	Not interactive; easy-to-follow text-based explanations with many colorful graphics; provides opportunity to select level of difficulty

URL=uniform resource locator.

Table 2.

Genetics Education Sites for Children/Young Adults

Site/URL	Authority	Special Features	Comments
Designer Genes *http://library.thinkquest.org/18258/index2.htm*	Created for the 1998 ThinkQuest Competition by students Jason Anderson, Ania Zaremba, and Chris Wedge	Comprehensive coverage of both basic and advanced genetics concepts, homework help, self quizzes; Polish version available	Not interactive; text supported with graphics, very detail oriented, geared toward more advanced students
Gene CRC—Kids Only *http://www.genecrc.org/site/ko/index_ko.htm*	The Cooperative Research Centre for Discovery of Genes for Common Human Diseases	Learn about basic genetics from characters GoGo Gene, Lucy, and Pipl interactive gene games with different difficulty levels; includes glossary	Easy-to-understand text-based explanations supplemented with graphics, interactive games for all ages
I Can Do That! *http://eurekascience.com/ICanDoThat/index.htm*	Eureka! Science Corp.	Cartoon characters (Gene, Polly, RayNA) take you on an informational tour of basic genetics, cells, and genetic methods	Not interactive, text supported wth graphics, narrative format captures user interest but information is very detailed
Just For Kids! A Cartoon Guide to Genetics *http://history.nih.gov/exhibits/genetics/kidsf.htm*	National Institutes of Health, Online Exhibit of the DeWitt Stetten, Jr, Museum of Medical Research	Slide show explaining key genetic concepts; includes links to other genetic resources	Not interactive; moving screen with graphics and simmple text presented in kid-friendly language; slide show may require a long time to download
Kids Genetics @ GlaxoSmithKline *http://genetics.gsk.com/kids/index_kids.htm*	GlaxoSmithKline	Profess U. Gene helps you learn about basic genetic concepts; includes interactive learning activities with different levels	Interactive learning activities are very engaging and make learning fun
Kids Health—What is a gene? *http://www.kidshealth.org/kid/talk/qa/what_is_gene.html*	The Nemours Foundation	Presents information on basic genetics and gene therapy; Spanish version available	Not interactive; simple, concise text-based explanations of genetic concepts without graphics
OLogy: The Gene Scene *http://ology.amnh.org/genetics/index.html*	American Museum of Natural History	Go on a genetic journey covering basic genetics, cloning, nature and nurtuer, and genetic engineering; includes self quiz, interactive games/activities	Visually stimulating exploration of genetics with innovative interactive learning sections
A Science Odyssey—DNA Workshop *http://www.pbs.org/wgbh/aso/tryit/dna/*	WGBH Boston (public broadcasting system)	Interactive learning activities for DNA replication and protein synthesis; includes glossary	Emphasis on DAN; less focus on basic genetics

Table 3.

Sites Describing the Genetics of Type 1 Diabetes

Site/URL	Authority	Special Features	Comments
Causes of Type 1 Diabetes *http://www.intelihealth.com/IH/ihtIH/WHIHW000/35132/35250/363533.html?d=dmtContent*	Aetna InteliHealth (Content reviewed by the Faculty of Harvard Medical School)	Focuses on putative genetic and environmental triggers of type 1 diabetes	Brief text-based discussion without graphics
Children Have Diabetes, Too (Chapter 3) *http://www.diabetic.com/children/chapter3page1.htm*	Diabetic.com	Online book covering general diabetes information and genetic susceptibility to type 1 diabetes	Easy-to-follow explanation geared toward families, supplemented with graphics
Genetics Education Program—Diabetes Fact Sheet *http://www.genetics.com.au/Genetics2004/PDF/FactSheets/34.pdf*	Centre for Genetics Education: New South Wales Dept of Health (Sydney, Australia)	Printable PDF fact sheet covering the genetics of type 1 and type 2 diabetes	Easy-to-follow text-based format with graphics of family tree (pedigrees)
Genetics of Type 1 (Autoimmune) Diabetes *http://joslin.org/research/genetics_type1.shtml*	Joslin Diabetes Center	Section at a diabetes site that provides information about the genetic basis of type 1 diabetes	Brief text-based discussion without graphics

DAMD17-01-1-0009

ANNUAL REPORT

1 NOV 03 - 31 OCT 04

APPENDIX 2:

GIFT-D ADDITIONAL MATERIAL

SIX ASSENT FORMS

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

ASSENT FOR A CHILD TO BE A SUBJECT IN A RESEARCH STUDY

Protocol Number: 03-131

Title: "Genetic Information for Testing Diabetes (GIFT-D)" the program component of the "New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes", Phase 1

Part 1- Assessment and Education 7-12 Assent

Principal Investigator and Telephone Number:

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Co- Investigators with Telephone Numbers:

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412-383-1407

Massimo Trucco, M.D.
Professor of Pediatrics, Children's Hospital of Pittsburgh
412-692-6570

Source of Support: Department of Defense

Initials and date

*What is this **research** study about and why is it being done?*

Diabetes is a disease children can get. You have a brother or a sister who has diabetes. We are trying to learn why certain children, like your brother or sister, get this disease.

You may have learned about genes in school. People with certain genes have chances to get certain diseases. We are trying to learn why certain children get diabetes and teach families how certain genes work.

What am I being asked to do?

We would like to show you a program about genes and diabetes. You will first be asked to answer questions on a computer. We will give you help if you need it. Then, you will see a short computer program on diabetes and genes. This visit should take no longer than 2 hours.

What are the benefits and risks of participating in this research study?

Doing this study can help you because you may learn more about genes and diabetes. Knowing more about your chances of getting diabetes can help you decide if you want to have your genes tested. The bad thing that could happen is that you find out about genes and diabetes and you may worry about your chance of getting diabetes. This could make you and your family upset.

Do I have to take part in this study and can I quit at any time?

You do not have to take part in this study and you can quit any time you want for any reason. Your decision to take part or not take part will not have any effect on your brother or sister's treatment at the clinic.

How will my privacy rights be protected?

We will make sure that no one other than the people testing you knows the answers to your questions or anything else about you.

Can I ask questions?

You can ask questions now about anything we have talked about. You can also ask questions any time during this study and we will be happy to answer them for you.

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

Do my parent(s) have to give their permission for me to take part in this study?

Yes, you parent(s) have to give permission for you to be in this study. They have their own form to sign. Both you and your parent(s) will receive a copy of this form after you have signed it.

Assent

I have explained the research to the child-subject in words and pictures that he/she understands. I believe that he/she understands the research and has assented to participation.

Signature of person explaining the research: _____

Printed name of person explaining research: _____

(For children who are developmentally able to sign name:)

This research has been explained to me, and I agree to participate.

Signature of child-subject: _____

Printed name of child-subject: _____

I believe that my child understands what this research involves and that he/she has given assent for his/her participation.

Signature of parent: _____

Investigator's Certification

I certify that this subject was not begun on any research component of this protocol until after this consent form was signed.

Date: _____ Investigator's signature: _____

_____
Initials and date

University of Pittsburgh
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ASSENT FOR CHILD TO BE A SUBJECT IN A RESEARCH STUDY

Protocol Number: 03-131

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Source of Support: Department of Defense

Initials and date

What is this research study about and why is it being done?

Diabetes is a disease that children and teenagers can get. You have a brother or a sister who has diabetes. We are trying to learn why certain children and teens, like your brother or sister, get this disease. You may have learned about genes in science class. People with certain genes have chances to get certain diseases. We are trying to learn why certain children get diabetes and teach families how certain genes work.

This study has two parts. The first part will be one visit. At that visit, you will be asked to answer some questions and do an online computer program. The second part will consist of one additional visit and a one month follow-up session on a home (or clinic) computer. There will be separate consent forms for each. This consent is for the first part only.

What am I being asked to do?

We would like to show you a computer program about genes and diabetes. You would be asked to answer some questions and do the online computer program. This visit should take no longer than 2 hours.

What are the benefits and risks of participating in this research study?

Doing this study can help you because you may learn more about genes and diabetes. Knowing more about your chances of getting diabetes can help you decide if you want to have your genes tested. The bad thing that could happen is that you learn more about genes and the chance of getting diabetes and become upset.

Do I have to take part in this study and can I quit at any time?

You do not have to take part in this study and you can quit any time you want for any reason. Your decision to take part or not take part will not have any effect on your brother or sister's treatment at the clinic.

How will my privacy rights be protected?

While we have taken every usual precaution (user ID's and Passwords) to protect you, there is always the chance that the website could be broken into. Although your information is entered using only a number and never a family name, it could happen that someone could recognize your family from the information found there.

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

Can I ask questions?

You can ask questions at any time, now or in the future.

Do my parent(s) have to give their permission for me to take part in this study?

Yes, your parent(s) have to give permission for you to be in this study. They have their own form to sign. Both you and your parent(s) will receive a copy of this form after you have signed it.

Assent

I have explained the research to the child-subject in words and pictures that he/she understands. I believe that he/she understands the research and has assented to participation.

Signature of person explaining the research: _____

Printed name of person explaining research: _____

This research has been explained to me, and I agree to participate.

Signature of child-subject: _____

Printed name of child-subject: _____

I believe that my child understands what this research involves and that he/she has given assent for his/her participation.

Signature of parent: _____

Investigator's Certification

I certify that this subject was not begun on any research component of this protocol until after this consent form was signed.

Date: _____ Investigator's signature: _____

_____
Initials and date

University of Pittsburgh
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Approval Date:
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IRB Number:

ASSENT FOR A CHILD TO BE A SUBJECT IN A RESEARCH STUDY

Protocol Number: 03-131

Title: "Genetic Information for Testing Diabetes (GIFT-D)" the program component of the "New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes", Phase 1

Proband Genetic Testing Assent 7-12

Principal Investigator and Telephone Number:

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Professor of Pediatrics, Children's Hospital of Pittsburgh
412-692-6570

Source of Support: Department of Defense

Initials and date

What is this research study about and why is it being done?

We are trying to learn why certain children, like you, get diabetes. You may have learned about genes in school. People with certain genes have chances to get certain diseases. Scientists can test people's spit to learn about their genes and their chances of getting certain diseases. We are trying to help you and your family to learn why you got diabetes.

What am I being asked to do?

We would like to test your genes to see why you got diabetes. You will be asked to swish some mouthwash and spit into a cup. If you don't like mouthwash, we can use a cotton swap and gently wipe some spit from the inside of your mouth. This should only take a few minutes. Your spit will be sent to a laboratory so that we can test your genes. By looking at your spit, we can help you to know more about why you got diabetes. This sample collection should only take a few minutes to do.

What are the benefits and risks of participating in this research study?

Studying your spit can help you and your family to learn why you got diabetes. We will look at the genes in your spit and in your brother's or sister's spit. We will see if there are some genes that are the same. We can help your family to know what the chances are for your brother or sister to get diabetes.

Giving the spit sample will not hurt you. Diabetes is not "catchy" but some children may worry that they can give it to their brother or sister. Learning more about genes can help children and families to know what their chances are of getting diabetes.

Do I have to take part in this study and can I quit at any time?

You do not have to take part in this study and you can quit any time you want for any reason. Your decision to take part or not take part will not have any effect on your treatment at the clinic.

How will my privacy rights be protected?

We will make sure that no one other than the people testing you knows the answers to your questions or anything else about you.

Can I ask questions?

You can ask questions now about anything we have talked about. You can also ask questions any time during this study and we will be happy to answer them for you.

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

Do my parent(s) have to give their permission for me to take part in this study?

Yes, you parent(s) have to give permission for you to be in this study. They have their own form to sign. Both you and your parent(s) will receive a copy of this form after you have signed it.

Assent

I have explained the research to the child-subject in words and pictures that he/she understands. I believe that he/she understands the research and has assented to participation.

Signature of person explaining the research: _____

Printed name of person explaining research: _____

(For children who are developmentally able to sign name:)

This research has been explained to me, and I agree to participate.

Signature of child-subject: _____

Printed name of child-subject: _____

I believe that my child understands what this research involves and that he/she has given assent for his/her participation.

Signature of parent: _____

Investigator's Certification

I certify that this subject was not begun on any research component of this protocol until after this consent form was signed.

Date: _____ Investigator's signature: _____

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Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
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IRB Number:

ASSENT FOR A CHILD TO BE A SUBJECT IN A RESEARCH STUDY

Protocol Number: 03-131

Title: "Genetic Information for Testing Diabetes (GIFT-D)" the program component of the "New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes", Phase 1

Proband Genetic Testing 12-17 Assent Form

Principal Investigator and Telephone Number:

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412-692-6570

Source of Support: Department of Defense

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

What is this research study about and why is it being done?

We are trying to learn why certain children, like you, get diabetes. You may have learned about genes in school. People with certain genes have chances to get certain diseases. Scientists can test people's spit to learn about their genes and their chances of getting certain diseases. We are trying to help you and your family to learn why you got diabetes.

What am I being asked to do?

We would like to test your genes to see why you got diabetes. You will be asked to swish some mouthwash and spit into a cup. If you don't like mouthwash, we can use a cotton swap and gently wipe some spit from the inside of your mouth. This should only take a few minutes. Your spit will be sent to a laboratory so that we can test your genes. By looking at your spit, we can help you to know more about why you got diabetes. This sample collection should only take a few minutes to do.

What are the benefits and risks of participating in this research study?

Doing this study can help you and your family to learn why you got diabetes. We will look at the genes in your spit and in your brother's and/or sister's spit. We will see if there are some genes that are the same. We can help your family to know what the chances are for your brother or sister to get diabetes.

Giving the spit sample will not hurt you. Diabetes is not "catchy" but some people may worry that they can give it to their brother or sister. Learning more about genes can help children and families to know what their chances are of getting diabetes.

How will my privacy rights be protected?

We will make sure that no one other than the people testing you knows your personal information. Your spit will be discarded as soon as we are done testing for your genes.

While we have taken every usual precaution (user ID's and Passwords) to protect you, there is always the chance that the website could be broken into. Although your information is entered using only a number and never a family name, it could happen that someone could recognize your family from the information found there.

Do I have to take part in this study and can I quit at any time?

You do not have to take part in this study and you can quit any time you want for any reason. Your decision to take part or not take part will not have any effect on your treatment at the clinic.

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

Can I ask questions?

You can ask questions now about anything we have talked about. You can also ask questions any time during this study and we will be happy to answer them for you.

Do my parent(s) have to give their permission for me to take part in this study?

Yes, you parent(s) have to give permission for you to be in this study. They have their own form to sign. Both you and your parent(s) will receive a copy of this form after you have signed it.

Assent

I have explained the research to the child-subject in words and pictures that he/she understands. I believe that he/she understands the research and has assented to participation.

Signature of person explaining the research: _____

Printed name of person explaining research: _____

(For children who are developmentally able to sign name:)

This research has been explained to me, and I agree to participate.

Signature of child-subject: _____

Printed name of child-subject: _____

I believe that my child understands what this research involves and that he/she has given assent for his/her participation.

Signature of parent: _____

Investigator's Certification

I certify that this subject was not begun on any research component of this protocol until after this consent form was signed.

Date: _____ Investigator's signature: _____

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ASSENT FOR CHILD TO BE A SUBJECT IN A RESEARCH

Protocol Number: 03-131

Title: "Genetic Information for Testing Diabetes (GIFT-D)" the program component of the "New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes", Phase 1

Part 2- Testing and Counseling 7-12 Assent

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Source of Support: Department of Defense

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

What is this research study about and why is it being done?

Diabetes is a disease that children can get. You have a brother or a sister who has diabetes. We are trying to learn why certain children and teens, like your brother or sister, get this disease. You may have learned about genes from our computer program. You know that scientists can test people's spit to learn about their genes and their chances of getting certain diseases. We are trying to learn why certain children get diabetes and teach families how certain genes work.
This study will be done in two parts. The first part is the education program on the computer. You already did that part. The second part has another visit and a one month follow-up session on a home (or clinic) computer. There will be separate consent forms for each. This consent is for the second part.

What am I being asked to do?

We showed you a computer program about genes and diabetes. You learned about genes, and how genes can be tested in your spit. We would like to test your genes to see if you have a chance of getting diabetes. You will be asked to swish some mouthwash and spit into a cup. If you don't like mouthwash, we can use a cotton swap and wipe some spit from the inside of your mouth. This should only take a few minutes. Your spit will be sent to a laboratory. By looking at your spit, we can help you to know more about your chances of getting diabetes.

You will then come back for another visit. You will watch a short program on the computer. You will be asked at the end of this program to decide whether or not you want to know about your chance for getting diabetes.

If you want to find out your chance, a counselor will meet with you. S/he will also be able to answer your questions. You will be able to find out what else you can learn about getting diabetes.

After that, you will again be asked to do some more questions. This visit should take less than 2 hours.

One month after this last visit, you will get a postcard asking you to go online to answer some more questions on the computer. You can do this at home, if you have a computer, or back in the clinic.

Initials and date

What are the benefits and risks of participating in this research study?

Doing this study can help you because you may learn more about genes and diabetes. Knowing more about your chances of getting diabetes can help you decide if you want to have your genes tested. The bad thing that could happen is that you find out that you may have a chance of getting diabetes. This could make you and your family upset.

You might not want to share things about yourself. We will make sure that everyone gets the same questions and we will not share your answers with anyone. If you do not want to answer the questions that is okay with us.

Do I have to take part in this part of the study and can I quit at any time?

You do not have to take part in this part of the study and you can quit any time you want for any reason. Your decision to take part or not take part will not have any effect on your brother or sister's treatment at the clinic.

How will my privacy rights be protected?

We will make sure that no one other than the people testing you knows your personal information. Your spit will be thrown away as soon as we are done testing for your genes.

Can I ask questions?

You can ask questions now about anything we have talked about. You can also ask questions any time during this study and we will be happy to answer them for you.

Do my parent(s) have to give their permission for me to take part in this study?

Yes, you parent(s) have to give permission for you to take part in this part of the study. They have their own form to sign. Both you and your parent(s) will receive a copy of this form after you have signed it.

Initials and date

University of Pittsburgh
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Approval Date:
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Assent

I have explained the research to the child-subject in words and pictures that he/she understands. I believe that he/she understands the research and has assented to participation.

Signature of person explaining the research: _____

Printed name of person explaining research: _____

(For children who are developmentally able to sign name:)

This research has been explained to me, and I agree to participate.

Signature of child-subject: _____

Printed name of child-subject: _____

I believe that my child understands what this research involves and that he/she has given assent for his/her participation.

Signature of parent: _____

Investigator's Certification

I certify that this subject was not begun on any research component of this protocol until after this consent form was signed.

Date: _____ Investigator's signature: _____

_____
Initials and date

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ASSENT FOR CHILD TO BE A SUBJECT IN A RESEARCH

Protocol Number: 03-131

Title: "Genetic Information for Testing Diabetes (GIFT-D)" the program component of the "New Advanced Technology to Improve Prediction and Prevention of Type 1 Diabetes", Phase 1

Part 2- Testing and Counseling 12-17 Assent

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412-383-1407

Massimo Trucco, M.D.
Professor of Pediatrics, Children's Hospital of Pittsburgh
412-692-6570

Source of Support: Department of Defense

Initials and date

What is this research study about and why is it being done?

Diabetes is a disease that children and teenagers can get. You have a brother or a sister who has diabetes. We are trying to learn why certain children and teens, like your brother or sister, get this disease. You may have learned about genes in science class. People with certain genes have chances to get certain diseases. Scientists can test people's spit to learn about their genes and their chances of getting certain diseases.
We are trying to learn why certain children get diabetes and teach families how certain genes work.
This study will be done in two parts. The first part is the education program on the computer. You already did that part. The second part has another visit and a one month follow-up session on a home (or clinic) computer. There will be separate consent forms for each. This consent is for the second part.

What am I being asked to do?

We showed you a computer program about genes and diabetes. You learned about genes, and how genes can be tested in your spit. We would like to test your genes to see if you have a chance of getting diabetes. You will be asked to swish some mouthwash and spit into a cup. If you don't like mouthwash, we can use a cotton swap and gently wipe some spit from the inside of your mouth. This should only take a few minutes. Your spit will be sent to a laboratory so that we can test your genes. By looking at your spit, we can help you to know more about your chances of getting diabetes.

You will then return for another visit to view a short program on the computer explaining genetic counseling. You will be given the opportunity at the end of this program to decide whether or not you wish to receive the results of your genetic risk for getting diabetes.

Should you decide to receive the results of the testing for your own risk (chance), a counselor will meet with you face-to-face to provide this information. S/he will also be able to answer your questions regarding diabetes and genetic risk. You will be given resources where you can obtain additional information should you wish to do so. You will also be informed about additional studies that are being done whose focus is the prevention type 1 diabetes.

Following this counseling session, you will again be asked to complete a brief series of questions on the web, much the same as those you completed on your first visit. This entire visit should take less than 2 hours.

Initials and date

One month after this last visit, you will receive a postcard in the mail asking you to go online to complete additional questionnaires and an evaluation of your experiences with this research project. You can complete this at home, if you have access to a computer, or back in the clinic. Your input will help us to provide the best possible program for future studies.

It is also our intention to contact you either by phone, letter or postcard in approximately 3 (and possibly 5) years to request that you complete a series of questionnaires online as a follow up to the initial research.

What are the benefits and risks of participating in this research study?

Doing this study can help you because you may learn more about genes and diabetes. Knowing more about your chances of getting diabetes can help you decide if you want to have your genes tested. The bad thing that could happen is that you find out that you may have a chance of getting diabetes. This could make you and your family upset.

You might also feel uncomfortable sharing personal information on questionnaires. To reduce this discomfort, we are using standard questionnaires on the computer and you will always have the option to not answer questions.

While we have taken every usual precaution (user ID's and Passwords) to protect you, there is always the chance that the website could be broken into. Although your information is entered using only a number and never a family name, it could happen that someone could recognize your family from the information found there.

Do I have to take part in this part of the study and can I quit at any time?

You do not have to take part in this part of the study and you can quit any time you want for any reason. Your decision to take part or not take part will not have any effect on your brother or sister's treatment at the clinic.

How will my privacy rights be protected?

We will make sure that no one other than the people testing you knows your personal information. Your spit will be thrown away as soon as we are done testing for your genes.

Can I ask questions?

You can ask questions now about anything we have talked about. You can also ask questions any time during this study and we will be happy to answer them for you.

Initials and date

University of Pittsburgh
Institutional Review Board
Approval Date:
Renewal Date:
IRB Number:

Do my parent(s) have to give their permission for me to take part in this study?

Yes, you parent(s) have to give permission for you to take part in this part of the study. They have their own form to sign. Both you and your parent(s) will receive a copy of this form after you have signed it.

Assent

I have explained the research to the child-subject in words and pictures that he/she understands. I believe that he/she understands the research and has assented to participation.

Signature of person explaining the research: _____

Printed name of person explaining research: _____

(For children who are developmentally able to sign name:)

This research has been explained to me, and I agree to participate.

Signature of child-subject: _____

Printed name of child-subject: _____

I believe that my child understands what this research involves and that he/she has given assent for his/her participation.

Signature of parent: _____

Investigator's Certification

I certify that this subject was not begun on any research component of this protocol until after this consent form was signed.

Date: _____ Investigator's signature: _____

_____
Initials and date

DAMD17-01-1-0009

ANNUAL REPORT

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APPENDIX 2:

GIFT-D ADDITIONAL MATERIAL

LETTER FROM UNIVERSITY OF MICHIGAN

THE UNIVERSITY OF MICHIGAN
SCHOOL OF PUBLIC HEALTH
PUBLIC HEALTH GENETICS PROGRAM

611 CHURCH STREET
ANN ARBOR, MICHIGAN 48104-3028

October 20, 2004

Dear HEB special issue participants:

Thank you again for your participation in last week's symposium at the University of Michigan. We have received many compliments on the event and hope that momentum gained can be shifted towards the special issue in *Health Education and Behavior* (HEB). In the sections below, we provide important details about your manuscript for the special issue.

The deadline for manuscript is November 30, 2004. Please see the attached timeline. We realize the timeline is very tight. However, in order to have the special issue in print by October 2005, the deadlines we've been provided with make this necessary. We appreciate the effort by everyone to help us make these deadlines. If we fail to meet the deadlines set by the Editor-in-Chief, the journal cannot guarantee the October 2005 issue for publication. Moreover, it is possible that the special issue would go to the end of the publication queue and could thereby significantly delay its publication.

There is a 25-page limit for all papers. This page limit ***includes*** title page, abstract, text, references, tables and figures. Manuscripts should have 1½" (3.8 cm) margins. The manuscript including captions, footnotes, tables, and references must be typed double-spaced. Footnotes in the text should be avoided where possible and no appendices included. APA reference styling is taking effect January 2005 for the journal and should be followed (for text and reference list) for all manuscripts included in the special issue.

The title page should contain the names of all authors followed by their degree, their affiliations, and a complete, current address, telephone number, fax number and electronic mail address. A footnote on the title page may contain simple statements of affiliation, credit, and research support. The names of the authors should not appear on the rest of the paper. Rather, a descriptive running head of no more than four words should appear on the top of each page of the manuscript.

An abstract of 100-150 words must accompany each submission. Please provide keywords in italic font after the abstract, i.e., Keywords: *first keyword, second keyword, third keyword.*

All papers must include a section in their discussion that addresses "Implications for Practitioners." Please see the journal copies we distributed at the symposium to help familiarize yourself with this section. Authors are asked not to use the term "subjects" when referring to research participants. Alternative terms such as respondents, research participants, or some other more specific designation (e.g., youths, females, residents) should be used.

The journal is in the process of switching the review process to a web-based all electronic review and submission system. Until that is operational, however, we are proceeding with the review process as

planned. As such, please email your manuscripts to Catharine Wang at clwang@umich.edu. We will inform you of any changes in the process when and if necessary.

We have included a copy of the Copyright Transfer Agreement for the journal. It must be completed and signed by all authors of each manuscript. No manuscript can be considered accepted unless a signed copyright transfer agreement exists.

Practice notes authors:

For Denise Charron-Prochownik/Jan Dorman and Hayley Thompson
Practice notes are 300 word descriptions of health education programs that do not include evaluation results. Format of the practice note includes the following headings: objective, assessment of need, strategy, evaluation approach, and implications for practitioners. A practice note can also include contact information that includes name, address, telephone number and email address if applicable. Please refer to the journal for examples of practice notes.

Please let us know if you have any questions. We are looking forward to receiving your manuscripts by the end of November.

Sincerely,

Catharine Wang, PhD
Guest Editor
University of Michigan

Deborah Bowen, PhD
Guest Editor
University of Washington

DAMD17-01-1-0009

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APPENDIX 3

RELEVANT PUBLICATIONS FROM THE PRINCIPAL INVESTIGATOR'S LABORATORY

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 2, Issue of January 10, pp. 853–863, 2003
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Alternative Core Promoters Regulate Tissue-specific Transcription from the Autoimmune Diabetes-related *ICA1* (ICA69) Gene Locus*

Received for publication, October 4, 2002, and in revised form, October 25, 2002
Published, JBC Papers in Press, October 29, 2002, DOI 10.1074/jbc.M210175200

Robert P. Friday‡§¶, Susan L. Pietropaolo‡, Jennifer Profozich‡, Massimo Trucco‡§, and Massimo Pietropaolo‡‖

From the ‡Division of Immunogenetics, Department of Pediatrics, Diabetes Institute, Rangos Research Center, Children's Hospital of Pittsburgh, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213 and §Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Islet cell autoantigen 69-kDa (ICA69), protein product of the human *ICA1* gene, is one target of the immune processes defining the pathogenesis of Type 1 diabetes. We have characterized the genomic structure and functional promoters within the 5′-regulatory region of *ICA1*. 5′-RNA ligase-mediated rapid amplification of cDNA ends evaluation of *ICA1* transcripts expressed in human islets, testis, heart, and cultured neuroblastoma cells reveals that three 5′-untranslated region exons are variably expressed from the *ICA1* gene in a tissue-specific manner. Surrounding the transcription initiation sites are motifs characteristic of non-TATA, non-CAAT, GC-rich promoters, including consensus Sp1/GC boxes, an initiator element, cAMP-responsive element-binding protein (CREB) sites, and clusters of other putative transcription factor sites within a genomic CpG island. Luciferase reporter constructs demonstrate that the first two *ICA1* exon promoters reciprocally stimulate luciferase expression within islet- (RIN 1046-38 cells) and brain-derived (NMB7) cells in culture; the exon A promoter exhibits greater activity in islet cells, whereas the exon B promoter more efficiently activates transcription in neuronal cells. Mutation of a CREB site within the *ICA1* exon B promoter significantly enhances transcriptional activity in both cell lines. Our basic understanding of expression from the functional core promoter elements of *ICA1* is an important advance that will not only add to our knowledge of the ICA69 autoantigen but will also facilitate a rational approach to discover the function of ICA69 and to identify relevant *ICA1* promoter polymorphisms and their potential associations with disease.

Islet cell autoantigen 69 kDa (ICA69) is identified with a group of Type 1 diabetes-related islet autoantigens considered to be specific protein targets of the diabetogenic autoimmune response. By using sera from pre-diabetic individuals, Pietropaolo and co-workers (1) first identified ICA69 through immunoscreening of a human islet cDNA expression library. The 1785-bp nucleotide sequence of the full-length clone and its deduced 483-amino acid protein coding region demonstrated no overt homology to known molecules at the time of its discovery, and nucleic acid and protein analyses revealed that the molecule is primarily expressed in neuroendocrine tissues (1, 2). More recent subcellular fractionation studies of murine brain tissue have shown that the majority of ICA69 protein is cytosolic and soluble, although a subfraction appears to be membrane-bound and associates with synaptic-like microvesicles (3). Although of unknown significance, smaller isoforms of the protein may be expressed from at least three human transcript variants (1, 4–6), representing truncated cDNAs that arise from alternative splicing of coding region exons (4).

ICA69 is encoded on human chromosome 7p22 by the *ICA1* gene (7), which is composed of 14 coding exons and three 5′-untranslated region (UTR)[1] sequences, each of which splices with exon 1 in a mutually exclusive manner (4). In addition, multiple cDNA coding region splice variants from human, mouse, and rat have been identified within islet and brain bacteriophage λ cDNA libraries by immunoscreening with human serum or by DNA probe hybridization (1, 4–6). Intron-exon boundaries were established for the human and murine *ICA1* genes using a combination of λ phage genomic DNA library screening and PCR experiments (4). Collectively, these data argue for a high level of evolutionary conservation of the *ICA1* gene, not only upon comparison of the human protein to the rat (6) and mouse (5) homologues but also in terms of exon/intron partitioning (4).

Recently a protein from the nematode *Caenorhabditis elegans*, termed ric-19, was reported to exhibit amino acid homology with ICA69 (3). Based on functional studies of ric-19 in *C. elegans*, these authors have proposed that ICA69/ric-19 participates in the process of neuroendocrine secretion through an association with secretory vesicles. These data suggest that ICA69 may be involved in the insulin secretory pathway in islet β cells, as the molecule is known to be specifically expressed within islets (8) and by insulin-producing cell lines maintained in culture (2). However, the true cellular function of ICA69 and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF146364, BZ286433, BZ286436, BAC503D2, BZ286434, BZ286435, YAC813G2, BZ286437, YAC813G2, and BZ286438.

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[1] The abbreviations used are: UTR, untranslated region; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; RLM, RNA ligase-mediated; CITB, the California Institute of Technology BAC Library; CEPH, the Centre d'Etude du Polymorphisme Humain; Inr, initiator; CREB, cAMP-responsive element-binding protein; NOD, non-obese diabetic; FF, firefly; RLU, relative light unit; NCBI, National Center for Biotechnology Information; HGP, Human Genome Project; GSP, gene-specific primers; TF, transcription factor.

its importance to normal mammalian pancreatic islet physiology remain unknown.

Most basic and clinical research investigations concerning ICA69 have focused on the importance of the molecule as an autoimmune target in Type 1 diabetes. Three lines of evidence from at least four independent groups substantiate a role for ICA69 autoimmunity in diabetes. 1) First degree relatives of diabetic patients who developed the disease during follow-up have detectable serum levels of ICA69 autoantibodies (1, 9). 2) T-cells isolated from newly diagnosed diabetic patients and from non-obese diabetic (NOD) mice demonstrate reactivity against the recombinant ICA69 molecule (10–12). 3) T-cells specific for the ICA69 peptide Tep-69 play a driving role in the acceleration of islet cell destruction in the NOD mouse model of Type 1 diabetes (13), whereas intraperitoneal injection of Tep-69 is associated with apparent immune toleration and decreased diabetes incidence in NOD mice (14). It has been reported that a majority of patients with recent onset Type 1 diabetes shows evidence of autoreactive T-cells and/or autoantibodies with immune specificity for the ICA69 molecule (11), but it must also be acknowledged that some investigators have questioned the significance of ICA69 autoantibodies based on their own studies (15).

Motivated by an interest in understanding how autoantigen expression in key body tissues relates to autoimmunity and as a prerequisite to searching for functional polymorphisms in the promoter region of the gene encoding ICA69, we have defined the basic structure and functional characteristics of the *ICA1* promoter. Sequences adjacent to the multiple *ICA1* transcription initiation sites contain motifs typical of a non-TATA, non-CAAT, GC-rich regulatory region, including consensus Sp1/GC box sites, Inr (initiator) elements, and CREB sites. The major alternative transcription initiation sites associate with independent 5′-UTR exons, and a detailed analysis of *ICA1* transcripts from different tissues provides evidence for a tissue-specific utilization of the distinct initiation sites consistent with the observed 5′-UTR heterogeneity of mature protein-coding ICA69 transcripts. *In vitro* luciferase reporter gene assays of promoter function correlate with the observed preferential transcription initiation site usage within different tissues, whereas site-directed mutagenesis of promoter reporter constructs demonstrate the importance of an Sp1/GC box site and a CREB site to the regulation of expression from exons A and B, respectively. The significance and potential implications of the *ICA1* promoter structure and function are discussed in the context of understanding ICA69 biology and its role as a Type 1 diabetes autoantigen.

EXPERIMENTAL PROCEDURES

Cell Lines—Two adherent cell lines were maintained in culture in order to provide RNA for transcript analysis and for testing promoter activity of cloned *ICA1* 5′-flanking sequences in a firefly luciferase reporter assay. The human neuroblastoma cell line NMB7 was grown in RPMI containing 10% fetal bovine serum, supplemented with L-glutamine and penicillin/streptomycin. Rat insulinoma (RIN 1046-38) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. Passage of cells was conducted at 70–80% confluence as necessary. All cell culture reagents were obtained from Invitrogen and lot-certified in-house. NMB7 cells were provided by Dr. Ira Bergman and Judi Griffin (Children's Hospital of Pittsburgh, Pittsburgh).

Nucleic Acid Purification—Human total genomic DNA was purified from whole blood using the Qiagen Genomic DNA Extraction Kit (Qiagen, Inc., Valencia, CA). Briefly, 10 ml of heparinized blood was successively subjected to cellular lysis, nuclear isolation, nuclear lysis, and anion-exchange chromatography using the buffers and prepared columns supplied with the Qiagen kit. Typical yields ranged from 150 to 350 μg of total genomic DNA per 10 ml of whole blood.

Bacterial plasmid DNA was isolated by one of two methods, depending upon the quantity and concentration desired. For DNA sequencing and restriction enzyme analysis of subcloned DNA, the QIAprep Spin Miniprep Kit (Qiagen) was used to isolate 10–20 μg of plasmid DNA from 3 to 5 ml of an overnight bacterial culture. Alternatively, plasmid DNA used for transfection of cultured cells was prepared from 100 to 200 ml of overnight bacterial culture using the Qiagen Maxiprep DNA Isolation Kit. Typical DNA yields ranged from 150 to 450 μg.

For BAC clone DNA, a Qiagen Maxiprep protocol modified for use with BACs was followed (protocol available from manufacturer). Major modifications to the basic protocol included the use of a larger culture volume (500 ml) and elution of BAC clone DNA from the column with buffer warmed to 50 °C. Yields of BAC clone DNA ranged from 100 to 150 μg.

YAC clone DNA was co-purified along with yeast genomic DNA according to a standard protocol (16) with some modifications. Briefly, following 2000 × *g* centrifugation of 100 ml of fresh yeast cell culture at room temperature for 10 min, the cell pellet was resuspended in SCE buffer (0.9 M sorbitol, 0.1 M sodium citrate, 0.06 M EDTA) with freshly added 0.3 M β-mercaptoethanol and lyticase enzyme (Sigma). Formation of yeast spheroplasts was allowed to proceed for 1–2 h at 37 °C with gentle shaking. Spheroplasted cells were then pelleted by centrifugation at 1000 × *g* for 10 min. Yeast cell lysis was achieved by suspension of spheroplasts in lysis buffer (0.5 M Tris-Cl, pH 8.0, 3% *N*-lauroylsarcosine, 0.2 M EDTA, 1 mg/ml proteinase K) buffer and incubation for 30–45 min at 65 °C in a water bath. Overnight treatment of cell lysate with RNase PLUS (5 Prime → 3 Prime, Inc., Boulder, CO) at 37 °C adequately removed contaminating yeast RNA from the sample. Isolation of the DNA fraction was achieved through two successive organic extractions with an equal volume of 50:50 phenol/chloroform, followed by 3–4 extractions of the aqueous phase with chloroform only. YAC and yeast DNA were co-precipitated from the aqueous phase with 0.1 volumes of 3 M NaCl and 2.5 volumes of ice-cold 100% ethanol. After gentle spooling, the precipitated DNA was washed in 70% ethanol and air-dried. Purified DNA was resuspended in TE buffer, pH 8.0, with a typical preparation yielding 300–700 mg of nucleic acid as measured by A_{260} measurement.

PCR Amplification—Due to the nature of the sequences being amplified, the PCR technology used in an experiment was adapted to each DNA target and template. Reaction components were used in amounts and concentrations as recommended by the manufacturer unless otherwise noted. Suggested annealing temperatures for each PCR kit were adjusted based on the sequence identity of the amplification primers and the sequence composition of the amplification target with the assistance of Oligo 4.0 (Molecular Biology Insights, Inc., Cascade, CO) primer design software. Reactions were cycled 30–35 times unless otherwise specified.

For PCR amplification of simple target DNAs (*i.e.* <5 kb with moderate to low GC content) Ampli*Taq* DNA polymerase enzyme and buffers (PerkinElmer Life Sciences/Applied Biosystems) were used. GC-rich regions of *ICA1* sequence from human genomic, YAC, and BAC DNA samples were amplified using reagents from the Advantage GC Genomic PCR kit (Clontech, Palo Alto, CA), whereas the Advantage GC cDNA Enzyme (Clontech) facilitated amplification of GC-rich plasmid inserts and cDNA templates generated for 5′-RACE analysis. In cases of long PCR (>5 kb), or when other PCR methods failed, the eLONGase long PCR enzyme mix (Invitrogen) was employed. Oligonucleotides were synthesized in the DNA Sequencing and Synthesis Core Facilities of the Diabetes Institute, Children's Hospital of Pittsburgh.

YAC and BAC Library Screening—PCR primers designed from the *ICA1* exon 2 intron-exon boundary sequences were used to screen the Centre d'Etude du Polymorphisme Humain (CEPH) Mega-YAC Human DNA Library by systematic amplification of YAC clone DNA pools (primer sequences: 5′-CCTGGGACTTACAGGATCGA-3′ and 5′-GACAGCAATAAAGAGCTCAC-3′, annealing temperature 55 °C, 178-bp PCR product). The California Institute of Technology BAC Library (CITB Release IV, Research Genetics, Inc., Pasadena, CA) was similarly screened using a PCR approach. The PCR amplimer used for BAC library screening was a microsatellite (CA repeat) centered 1830 bp upstream of the *ICA1* translation initiation codon (primer sequences: 5′-TATGAAACAGTGTTATTCTGGACCT-3′ and 5′-GTACAGTATAGTAGTGCTAACA-3′, annealing temperature 55 °C, 540-bp PCR product). Stab vials or frozen aliquots of each PCR-positive YAC and BAC library clone identified through screening were obtained, and purified DNA extracted from their respective cultures was retested under PCR conditions similar to those used for library screening to verify that the target *ICA1* sequences were present.

Subcloning of PCR Products—If necessary, amplified products from GenomeWalker-PCR, RT-PCR, and 5′-RACE experiments were gel-

purified using the Qiagen Gel Extraction Kit (Qiagen) before subcloning, or they were subcloned by direct ligation of an aliquot of the PCR. The gel-purified or neat PCRs were subcloned into the pCR 2.1 vector (Original TA Cloning Kit, Invitrogen). When PCR primers were designed to include restriction sites, they were digested with the appropriate restriction enzyme(s) and ligated into an overhang-compatible aliquot of the pGL3 basic luciferase reporter vector. All ligation reac-competent *Escherichia coli* (Invitrogen) and plated on 100-mm LB agar plates containing 50 μg/ml ampicillin or kanamycin and 50 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal) for blue-white color selection of transformants (if applicable).

In situations where T/A PCR product ligation proved to be inefficient because of 3′→ 5′-exonuclease activity from the PCR enzyme or enzyme mix used for amplification, the PCR product(s) were "tailed" with dA-overhangs prior to ligation. Briefly, 1 unit of Ampli*Taq* DNA polymerase was added to the completely cycled PCRs, incubated for 15 min at 37 °C, and immediately extracted with an equal volume of phenol/chloroform. The tailed products were then ethanol-precipitated, resuspended in a small volume (~½ of original reaction volume), and directly used in the T/A ligation step.

5′-Rapid Amplification of cDNA Ends (5′-RACE)—The FirstChoice RLM-RACE Kit (Ambion, Austin, TX) was used for RNA ligase-mediated (RLM) RACE analysis of *ICA1* transcripts, because it permits selective amplification of capped RNA molecules from non-poly(A)-selected RNA. Briefly, total cellular RNA is treated with calf intestinal alkaline phosphatase to remove the 5′-phosphate group from uncapped mRNA precursors, tRNA, rRNA, and small nuclear RNA molecules, followed by phenol/chloroform extraction and recovery of the dephosphorylated RNA by ethanol precipitation. Dephosphorylated RNA is then incubated with tobacco acid pyrophosphatase to remove m^7Gpp from the cap structure of the 5′ end of capped RNAs, leaving a single 5′-terminal phosphate group. Ligation of a synthetic RNA adapter of known sequence to the calf intestinal alkaline phosphatase- and tobacco acid pyrophosphatase-treated RNA proceeds in the presence of *E. coli* RNA ligase. Adapter-ligated RNA is reverse-transcribed into cDNA using Moloney murine leukemia virus-reverse transcriptase enzyme in the presence of random decamer primers. The resultant single-stranded cDNA then serves as template in nested PCRs using adapter sequence-specific primers (provided with the RLM-RACE kit) and gene-specific primers (GSP1 and GSP2) designed from *ICA1* exons 1 and 2. The sequences of these latter primers are as follows: GSP1 (antisense exon 2), 5′-TGCATCTTATTTACAACTGACTTATCTTGA G-3′ and GSP2 (antisense exon 1/2 boundary), 5′-TGTAAGTCCCAGGGATAACTGCATTTGTGT CCTGA-3′. The Advantage GC cDNA enzyme was used in all nested RLM-RACE PCRs.

Cloning of ICA1 Sequences into pGL3 Basic—To clone segments of the *ICA1* 5′-flanking region and UTR exons, a 1028-bp genomic segment spanning the entire region was amplified from CITB-503D2 DNA via PCR, followed by T/A ligation of the product into the pCR2.1 vector (amplification primers, 5′-TAGGAAGCAGCTATGCCAACACT-3′ and 5′-CAGAGAAGGCAGCTCCTACCA-3′). Excision of various segments of the cloned PCR product using pairs of restriction endonucleases recognizing sites found in the pCR2.1 vector arms, internal restriction sites of the insert, or a combination of the two allowed for directional cloning of defined *ICA1* sequences into a pGL3 Basic vector having compatible overhangs. A second strategy for cloning *ICA1* sequences into pGL3 Basic involved the design of *ICA1*-specific primers with restriction endonuclease sites added at the 5′ end. After spin column chromatography purification of a PCR product amplified with these primers, the product was digested with one or more restriction enzymes to create overhangs compatible with those generated on an aliquot of the pGL3 Basic vector. Heat inactivation and gel purification or spin column purification of the digested PCR product was then followed by ligation into pGL3 Basic.

Site-directed Mutagenesis—Two pGL3 promoter reporter constructs, ExA −957 and ExB −440, were modified using the QuikChange Site-directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) to introduce mutations at suspected key sites within these promoters. Sequence mutations were introduced to the Sp1/GC box, Inr, (Sp1/GC box + Inr), and CREB sites within ExA −957 using the following oligonucleotides, respectively (mutant name and bases mutated are in boldface and in parentheses): 5′-CCTGCCGGAGAGCAGGG**tatt**GGTCACTCTGGGCGGCG (ExA-GC, −564 to −561), 5′-CGGAGAGCAGGGGCGGGGT**gg-agg**TGGGCGGCGGATCCG (ExA-Inr, −557 to −553), 5′-CCTGCCGGAGAGCAGGG**tatt**GGT**ggagg**TGGGCGGCGGATCCGAGC (ExA-GC-/Inr, mutations of −564 to −561 and −557 to −553), and 5′-CCTGTCCGCCAGGTCAT**cggc**ACGCAAACGCTATGGCCACGTGG (ExA-CREB, −612 to −609). For the ExB −440 construct, the Sp1/GC box and CREB sites were modified with the following oligonucleotides, respectively: 5′-CCGGTTCCTGCGCTCCCC**aata**CCCTTTCCCTCGCCTTCG (ExB-GC, −196 to −193) and 5′-CCCTTTCCCTCGCCTTCG**atcc**ACGCTGACGTCGGATGAGTG (ExB-CREB, −174 to −171). The mutation strategy of the QuikChange protocol was adhered to for all site-directed mutation reactions, using the above oligonucleotides in combination with a reverse complement sequence primer in each PCR-based mutagenesis reaction. After digestion of the reactions with *Dpn*I to remove non-mutated, methylated DNA, each mutated plasmid reaction was used to transform XL1-Blue supercompetent cells. Resultant colonies were then miniprepped and screened via automated fluorescent sequencing for successful mutation incorporation.

Luciferase Assays—For luciferase transfection experiments, NMB7 and RIN 1046-38 cells were plated at a density of 0.8×10^5 cells/well of a 12-well plate the day before transfection. Growth in the appropriate complete medium for 20–24 h generally resulted in 50–70% cellular confluence in each well at the time of transfection. Transient transfection of luciferase constructs and mutants thereof into the various cultured cell lines using Effectene Transfection Reagent (Qiagen) was followed by incubation of the transfected cells at 37 °C and cellular lysis 35–45 h after transfection. Luminescence assays of cellular lysates allowed for a semi-quantitative measure of luciferase production driven by each cloned segment of the *ICA1* 5′-flanking region. Within a given assay, plate wells were set up in triplicate for each transfected construct or control vector. The amount of DNA transfected was held constant for each construct and cell line, with a total amount 0.3 μg/well of a 12-well plate. Each Effectene reagent was used in the amount recommended by the manufacturer's protocol in proportion to the amount of DNA applied to each well. The strength of the promoting activity for each construct was assessed by comparison to basal luciferase expression from the promoterless pGL3 Basic vector transfected into triplicate samples of the same cell type within the same assay. To allow for normalization of firefly luciferase values based on transfection efficiency, a co-reporter vector expressing *Renilla* luciferase from the thymidine kinase promoter (pRL-TK) was included at a ratio of 1:10 of co-reporter plasmid to experimental promoter construct (or control vector) in the transfection mixture. Careful optimization of transfection conditions to maximize transfection efficiency provided an assay system yielding consistent results from repeated experiments.

Transfected cells were lysed by adding 100 μl of Passive Lysis Buffer (Promega, Madison, WI) to each well of a 12-well plate, followed by vigorous pipetting of the detached cells. Cell lysates were subjected to two freeze-thaw cycles (liquid N_2 and 20 °C H_2O) and either immediately assayed for luciferase activity or stored at −70 °C for analysis the following day. Firefly and *Renilla* luciferase activities of each lysate were measured sequentially via manual reagent injection in a Monolight 2010 luminometer using the Dual-Luciferase Reporter Assay System (Promega).

In order to compare inter-construct firefly (FF) luciferase activity values, the raw data relative light unit (RLU) readings were corrected by normalizing each sample according to transfection efficiency. One *Renilla* luciferase RLU (R-RLU) measurement from the pGL3 Basic control transfectant samples in a given experiment was selected and used to normalize each measured FF luciferase value as follows: ((normalizing R-RLU) ÷ (sample R-RLU)) × (sample FF-RLU) = (Nml sample FF activity). The normalized (Nml) triplicate values for each construct were then averaged to arrive at a relative measure of luciferase activity for that *ICA1* promoter reporter construct. Fold increases in promoter activity over the pGL3 Basic vector were calculated from the following formula: (Avg Nml sample FF activity) ÷ (Avg Nml pGL3 Basic control) = (sample fold increased activity over pGL3 Basic); where Avg is average. These calculations were performed independently for each transfection experiment data set (n = 3–5), and the average of all results obtained for a given *ICA1* promoter reporter construct was used as a measure of relative promoter strength. Where indicated, statistical analysis of luciferase reporter data was performed using the Mann-Whitney U test.

Oligonucleotide Synthesis—All oligonucleotides used as primers in the various PCR-based methods were synthesized on an ABI 394 DNA Synthesizer (Applied Biosystems, Inc.) using solid phase synthesis and phosphoramidite nucleoside chemistry, unless a primer was provided with a particular molecular biology kit.

Automated Fluorescent Sequencing—Automated fluorescent sequencing of plasmid DNA or purified PCR products was performed using an ABI 377 Automated DNA Sequence Analyzer (Applied Biosystems, Inc.) with either the dRhodamine or BigDye Terminator Cycle Sequencing Kits (Applied Biosystems, Inc.). Typically, TA vector-cloned PCR products were sequenced using the universal −21 M13 and M13

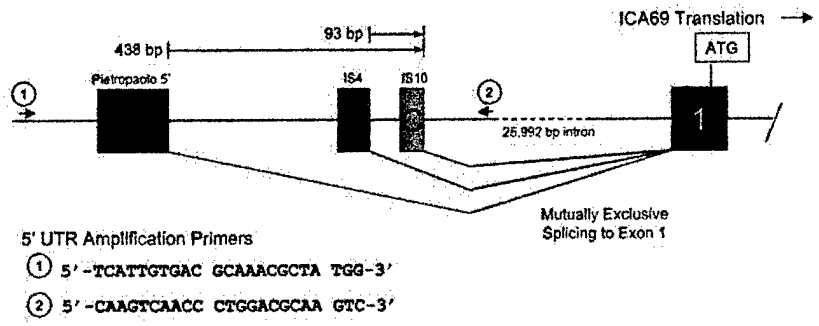


FIG. 1. **Organization of the *ICA1* 5'-UTR exons.** Flanking PCR primers were used to amplify the genomic region containing *ICA1* 5'-UTR exons from YAC and BAC clone DNA. Because of the high GC content of the region, specialized GC-rich PCR conditions were employed. Amplified PCR products were directly sequenced using the indicated amplification primers, confirming the UTR exon arrangement shown. The three 5'-UTR exons localize >26 kb upstream of the translation initiation codon in exon 1 yet span a genomic interval of <600 bp. The three exons are not found spliced to one another in any combination within isolated *ICA1* cDNAs or ESTs, indicating that the splicing to exon 1 is mutually exclusive.

reverse primers or internal primers designed from insert sequences. Direct sequencing of PCR products involved centrifugal filtration purification of the amplified DNA on Amicon Microcon YM-50 columns (Millipore Corp.) followed by sequencing with the same primers used for amplification. Inserts contained within the pGL3 Basic vector were sequenced with primers designed from regions flanking the multiple cloning site of the vector (pGL3-upstream, 5'-AGTGCAAGTGCAGGTGCCAG AA-3' and pGL3-downstream, 5'-CTTTATGTTTTTGGCGTCTTCCAT-3') or with primers internal to the cloned *ICA1* sequence.

Sequencing of YAC and BAC Clone Ends—To sequence YAC clone ends, the junctions between the two YAC vector arms and the insert sequence were first amplified and subcloned from a YAC fragment library constructed using the Universal GenomeWalker (GW) kit (Clontech Laboratories, Inc.). A gene-specific primer set (GSP1 and GSP2) was designed for each of the YAC vector arms to be used in combination with the set of nested GenomeWalker adapter primers provided with the GenomeWalker kit: HYAC-C, 5'-GCTACTTGGAGCCACTATCGACTACGCGAT-3' and LS-2, 5'-TCTCGGTAGCCAAGTTGGTTTAAGG-3' (left YAC arm); HYAC-D, 5'-GGTGATGTCGGCGATATAGGCGCCAGCAAC-3' and RA-2, 5'-TCGAACGCCCGATCTCAAGATTAC-3' (right YAC arm). Reaction conditions suggested by the GenomeWalker kit were employed without modification. Any PCR products amplified from the five YAC GenomeWalker library reactions were subcloned into the pCR2.1 vector for plasmid-based automated fluorescent sequencing.

The ends of each BAC clone were sequenced directly using 1 μg of purified BAC clone DNA in each of two automated fluorescent sequencing reactions extended from the two universal primers −21 M13 and M13 reverse.

Sequence Homology Analyses—Homology searches of nucleic acid and protein amino acid sequences were conducted through the Basic Local Alignment Search Tool (BLAST) server available on the National Center for Biotechnology Information (NCBI) internet website (www.ncbi.nlm.nih.gov).

GenBank™ Sequence Submissions—Novel *ICA1* cDNA and promoter function-associated genomic regions were submitted to the GenBank™ data base using the BankIt submission tool available through the NCBI website. YAC and BAC clone end sequences were submitted to the GSS data base via electronic mail to the address: batch-sub@ncbi.nlm.nih.gov.

Transcription Factor Binding Site Analysis of 5'-Flanking Sequences—To assess the 5'-flanking and UTR regions of *ICA1* for potential regulatory sequences, genomic DNA sequences of interest were analyzed using the public domain MatInspector version 2.2 software program available on the internet (genomatix.gsf.de/cgi-bin/matinspector/matinspector.pl). A core similarity of ≥0.900 from the MatInspector analysis was used as a cut-off for consideration of potential query sequence matches with known transcription factor recognition sequences.

RESULTS

Genomic Organization of the ICA1 5'-UTR Exons—We identified a single *ICA1*-containing CEPH Mega-YAC clone (CEPH-813G2) in a PCR-based YAC library screen. By using DNA purified from this YAC clone, we then initiated a cloning strategy involving primer-based genome walking in the 5' direction from *ICA1* exon 1, but we failed to identify any *ICA1* 5'-untranslated sequence within ~12 kb of genomic DNA sequence upstream of the translation initiation codon (data not shown). We did, however, identify a microsatellite (18–20 adjacent CA dinucleotides) within this interval (~1830 bp upstream of the ATG), for which we designed flanking primers that uniquely amplify this marker locus from CEPH-813G2 DNA as well as from human genomic DNA. The microsatellite flanking primers were used in a successful PCR-based screen of the California Institute of Technology BAC (CITB) library. Two BAC clones, CITB-426N6 and CITB-503D2, were identified as PCR-positive for the expected microsatellite amplimer band.

Sequence from a Human Genome Project (HGP) PAC clone (RP11-560C1, GenBank™ accession number AC007009, R.H. Waterston, Genome Sequencing Center, Washington University School of Medicine, St. Louis, MO) that encompasses all three of the known *ICA1* 5'-UTR sequences became publicly available shortly after we had identified the two *ICA1*-positive BAC clones. We confirmed these data (Fig. 1) by amplifying and directly sequencing PCR products spanning the region of interest from our YAC and BAC clone DNA using flanking primers. It must be noted that we have labeled the *ICA1* 5'-UTR exon sequences as exons A, B, C rather than adopt the −3, −2, −1 exon notation proposed by Gaedigk *et al.* (4). This modification to the exon identifiers was made because the genomic alignment of the UTR exons reported by these authors is in error, as they had localized the true leading exon (exon A or exon −2) between exons B (exon −3) and C (exon −1) in their report of the exon-intron boundary data. We feel that maintaining the negative integer notation for these exons would lead to confusion regarding the overall genomic organization and promoter structure of the *ICA1* gene.

The *ICA1* gene locus within its chromosomal context is summarized in Fig. 2, demonstrating that the *ICA1* gene is transcribed from 7p22 in a Tel→Cen orientation. The BAC clones CITB-426N6 and CITB-503D2 that we identified share significant overlap with respect to their genomic sequence content (according to BAC end sequencing results), and together they span a gap that had once existed between HGP clones RP11–560C1 and RP4–594A5. It is also notable that the RP11–560C1 PAC clone insert begins ~10 kb to the 5' side of *ICA1* exon A and extends downstream to include every *ICA1* exon with the exception of exon 14. Sequence from the YAC clone ends has allowed us to map our data onto HGP sequence data, with the YAC insert spanning 1.09 Mb of the HGP chromosome 7 working draft sequence (Fig. 2). The entire *ICA1* locus is contained within this YAC clone, along with nearly 1 Mb of downstream sequence extending centromeric from 7p22.

Analysis of cDNAs and ESTs from the ICA1 5'-UTR—To characterize the *ICA1* gene transcription initiation site(s), we first examined ICA69 cDNA and EST sequences available in

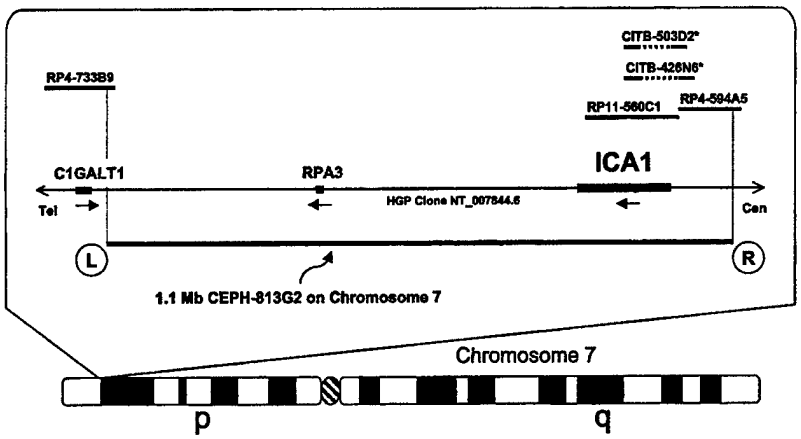


FIG. 2. **Chromosomal context of isolated YAC and BAC clones at the *ICA1* locus.** CEPH(Mega YAC)-813G2 intercepts a 1.1-Mb sequence mapping to HGP chromosome 7 clone NT_007844.6. The two PAC clones RP4-733B9 (accession number AC005532) and RP4-594A5 (accession number AC007128) define this interval, as they yielded positive BLAST hits for left (*L*) and right (*R*) CEPH-813G2 end sequences, respectively. The relationship of the two BAC clones from our library screen (CITB-426N6 and CITB-503D2) to PAC clones RP11-560C1 (accession number AC007009) and the *ICA1* locus was determined by BAC end sequencing and is also illustrated. The only other defined gene encompassed by the YAC sequence is RPA3 (replication protein A3), although C1GALT1 (UDP-galactose:*N*-acetylgalactosamine-α-R β1,3-galactosyltransferase) lies within 1 Mb telomeric to the start of *ICA1* immediately beyond the end of the YAC clone insert. The direction of transcription for each of these genes is indicated by an *arrow below* the chromosome 7 clone NT_007844.6 sequence. Hypothetical genes derived from computer analysis of genome data are not included in the diagram; however, it should be noted that a high concentration of putative protein coding segments flanks the left end of the YAC clone, whereas the remainder of the interval is rather sparsely populated by potential genes or ESTs found in the data base.

the NCBI GenBank™ data bases for messages with potentially full-length 5′ ends. As shown in Fig. 3, publicly available human *ICA1* transcripts generally do not agree with respect to the sequence content and/or length of their 5′-untranslated leader regions. Three species of mRNA differing in 5′ end sequence content are found, corresponding to the splicing of the known exon A, B, and C sequences downstream to either of two splice acceptor sites in *ICA1* exon 1 common to all transcripts. Splicing to exon 1 is variable, with some exon B transcripts using a splice acceptor farther downstream than the splice site found in all exon A transcripts.

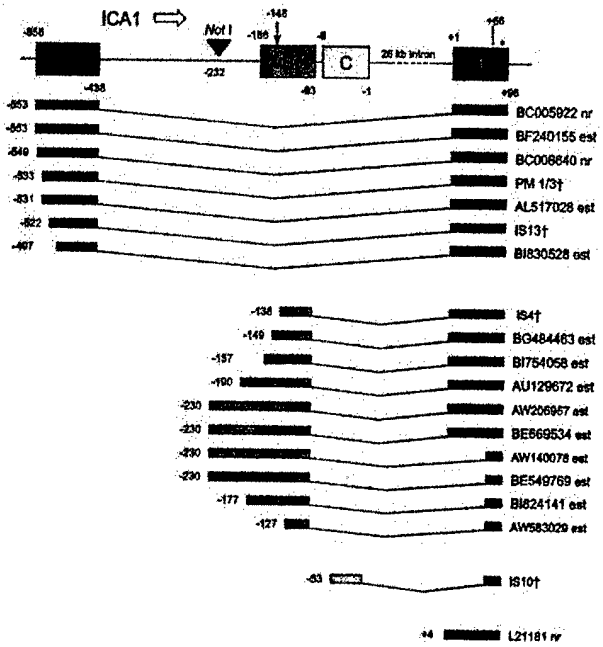
Additionally, there is great variability in the 5′ termini of sequences from each of the exons depicted in Fig. 3. Notably, the 5′ exon B untranslated sequences from cDNA clone IS4 and ESTs AW583029, BG484463, and BI754058 are short by comparison with the ESTs truncating at the *Not*I restriction site, whereas the exon A clones variably terminate over a range of 56 bp. The extension exon B transcripts to the *Not*I restriction site is likely to be an artifact of the EST library preparation method, which commonly employs this rare cutter to generate sticky ends for cloning, so the 5′ termini of these clones have been cleaved off.

RLM-RACE Localization of the ICA1 Transcription Initiation Sites—We identified transcription initiation sites and determined the lengths of *ICA1* exons A–C by analyzing islet, testis, heart, and NMB7 total cellular RNA via RNA ligase-mediated 5′-RACE (RLM-RACE). Gene-specific primers (GSPs) for amplification of *ICA1* 5′-UTRs were designed from the sequences of exons 1 and 2. Because these exons are common to all ICA69-encoding transcripts, different *ICA1* 5′ end sequence clones could be sequenced individually regardless of the amplified UTR flanked by the known RLM-RACE adapter and exon 1/2 sequence primers.

The results of our RLM-RACE analysis are summarized in Fig. 4. For each RNA sample analyzed at least 20 RACE-PCR-generated clones were independently sequenced. Overall, significant agreement of the first nucleotide from the sequenced exon A and B transcripts is noted both within and among the different tissues. Although every sequenced clone beginning with exon A or exon B did not start at exactly the same nucleotide, a cluster of start sites within a 2–4-bp interval was consistently detected for the major species from each exon. Additional variability in the exon A starting nucleotide was observed for testis transcripts, as two clones extended beyond the major start site and three clones proved to be shorter. The alternative starting nucleotides for exon B transcripts appear to be utilized in a somewhat tissue-specific manner. Most notably, every RLM-RACE clone derived from heart tissue transcripts agrees with regard to 5′-UTR exon length and starting 5′-nucleotide, extending ~35 nucleotides upstream of the common exon B start site identified from testis, islet, and neuroblastoma RNA samples.

Complete Genomic Organization of the ICA1 Gene—By having defined the sizes of the *ICA1* 5′-UTR exons, our knowledge of *ICA1* exon-intron organization is now complete (Table I). The genomic interval from the RLM-RACE determined transcription initiation site for exon A through coding exon 14 spans >148.7 kb as calculated from available chromosome 7 HGP data. Several of the intron distances determined from these data differ significantly in comparison to the PCR-amplified size data reported by Gaedigk *et al.* (4). Of note, the length of the intron between exon C and exon 1 as well as the lengths of introns 2, 6, and 8 were previously unknown. Remarkably, intron 6 is 59,651 kb long based on clone RP11-560C1 data, comprising 40.1% of total *ICA1* gene length. Significant refinements of intron lengths over the previous report are noted for introns 3, 7, 12, and 13. For example, the length reported for intron 3 by Gaedigk *et al.* (4) was 6.5 kb; however, we confirmed the length of this intron to be only 749-bp by amplifying and directly sequencing the intron with primers designed from exons 3 and 4 (data not shown).

Defining ICA1 Promoter Function—Our approach to analyzing the *ICA1* 5′-flanking sequences for promoter activity was instructed by the confirmation of initiation sites for transcription of the *ICA1* gene and assisted in part by a computer analysis of the flanking sequences for potential transcription activator sequences and transcription factor (TF)-binding sites. The sequence of this region and the locations of TF clusters are presented in Fig. 5. Fig. 5 includes the entire sequence of interest from the functional *ICA1* 5′-flanking region, with the UTR exon sequences and major transcription initiation sites identified. Clusters of high scoring sequence matches to TF-



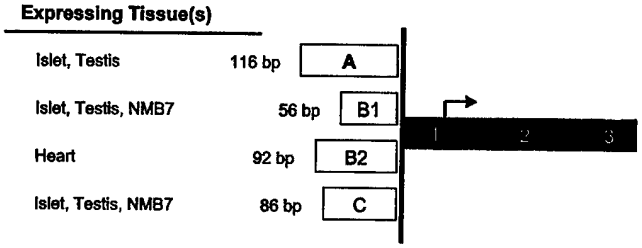
† KEY TO PUBLISHED cDNA CLONES

Name (bp)	GenBank Acc.	Reference
PM 1/3 (1785)	L01100	Pietropaolo, 1993
IS13 (1427)	U38260	Gaedigk, 1996
IS4 (1964)	U26592	Gaedigk 1996
IS10 (2139)	U26591	Gaedigk 1996

FIG. 3. **cDNA clones and ESTs containing *ICA1* 5′-UTR exon sequence.** A BLAST search of the non-redundant (NR) and human EST data bases using genomic sequence from the 5′-UTR region and exon 1 returned 19 matches for sequences demonstrating splice patterns consistent with *ICA1* transcripts as indicated by downstream splicing to exon 2 (not shown). Alignment of these cDNA and EST clones does not clearly define a consensus for initiation sites among the three UTR exons. Although three of the exon A clones approach the size for this exon as determined from our data (as per Figs. 4 and 5), these sequences were not obtained with the purpose of defining transcription initiation sites for the gene. For exon B containing transcripts, the apparent agreement of sequences terminating at −230 is likely an artifact related to the existence of a *NotI* restriction site at this location. The remaining exon B clones exhibit little agreement in their 5′ termini. The numerical annotation adopted for this figure is consistent with that used in the text and is arbitrarily defined by designating the last base of exon C as −1. All of the untranslated sequences are numbered negatively from this point upstream along the genomic sequence continuum. The first base of exon 1 is numbered +1. An internal exon 1 alternative splice acceptor site occurs around +66, and an *asterisk* denotes the location of the translation initiation codon at +80 relative to this splice acceptor site.

binding sites (MatInspector version 2.2 results) are arrayed schematically in Fig. 6*A* to emphasize the high density of potential regulatory elements in proximity to the three major *ICA1* transcription initiation sites. A close inspection and analysis of the sequence also reveal that it meets criteria to define a CpG island, specifically being a sequence tract of >200 bp with GC content of >50% and an observed:expected ratio for the occurrence of the dinucleotide CG [O:E(CpG)] of >0.6 (17). For a 1000-bp segment of *ICA1* 5′-UTR flanking region extending downstream from base −680, the sequence is composed of 73.9% GC bases and has an O:E(CpG) ratio of 0.81.

The delineation of independent *ICA1* 5′-UTR exons required that our promoter cloning strategy address the possibility that independent promoter activities are associated with the three separate exons. Thus, we constructed four basic luciferase promoter reporter plasmids (Fig. 6*B*). The first includes upstream flanking sequence contiguous with a downstream sequence interval encompassing all three 5′-UTR exons (ExABC −1012,



Tissue or Cell Line	Exon A		Exon B		Exon C	Exon 1*	Total n†
	-578 or -577	-556 or -555	-186	-151 to -148	-86 or -38	+57 to +66	
Testis	2	11	—	5	2	2	25
Islets	1	10	—	9	2	8	30
Heart	—	—	20	—	—	—	20
NMB7	—	—	1	14	4	1	22

FIG. 4. **Summary of RLM-RACE results for *ICA1* initiation.** Four different sources of RNA were assayed to determine the frequency of use for the 5′-UTR exons in *ICA1* transcription initiation. Four major transcript variants (exons A, B1, B2, and C) that include sequence from the 5′-UTR exons were identified. Exon A and exon B transcripts were more highly represented overall as compared with exon C transcripts. Notably, exon A transcripts were absent from the set of neuroblastoma and heart *ICA1* transcripts sequenced, whereas the heart transcript set was dominated by the expression of the longer exon B2 transcript variant. The *asterisk* indicates that for transcripts appearing to begin in exon 1, there was no one dominant starting nucleotide and that this region is the same region in which alternative splice acceptance of 5′-UTR exons occurs. The † signifies that the *"Total n"* reflects a larger number of sequences than those included in the table. The remaining few sequences variably terminated at points within exons A and B but without an apparent pattern.

TABLE I
Summary of ICA1 exon-intron lengths and genomic organization

Exon	Exon size	Intron	Intron size[a]	Intron as percentage of total locus size
	bp		*bp*	
A	116			
B	56 or 92	A-B	290 or 254	0.19
C	38 or 86	B-C	56 or 8	0.01
1	96	C-1	25,992	17.47
2	166	1	3,154	2.12
3	73	2	3,916	2.63
4	214	3	749	0.50
5	124	4	6,239	4.19
6	199	5	2,771	1.86
7	126	6	59,652	40.10
8	99	7	1,312	0.88
9	98	8	13,139	8.83
10	53	9	2,057	1.38
11	63	10	2,717	1.83
12	42	11	103	0.07
13	270	12	10,697	7.19
14	272	13	13,828	9.29
Totals	2,152		146,623	98.55%

[a] Underlined values represent major corrections of previous size estimates (4), based on HGP data and/or PCR amplification and sequencing in our laboratory. Intron 3 was amplified and directly sequenced using the following PCR primers: 5′-CAGAGAACCTGTCTGGACTT-3′ (exon 3) and 5′-CACAACGGCTCATTATAGGTC-3′ (exon 4).

total plasmid insert length 1031 bp). The three remaining reporter plasmids contain sequences from only one of the 5′-UTR exons, along with a reasonable amount of upstream flanking sequence truncated so as not to include any portion of the preceding exon (ExA −957, 453 bp; ExB −440, 293 bp; and ExC −90, 109 bp). The ExA −957 and ExB −440 constructs were engineered to exclude the splice donor site at their 3′ termini so that cryptic splicing events would be minimized during transcription of the luciferase reporter gene *in vivo*. The two cell lines used in our promoter reporter assays were chosen based

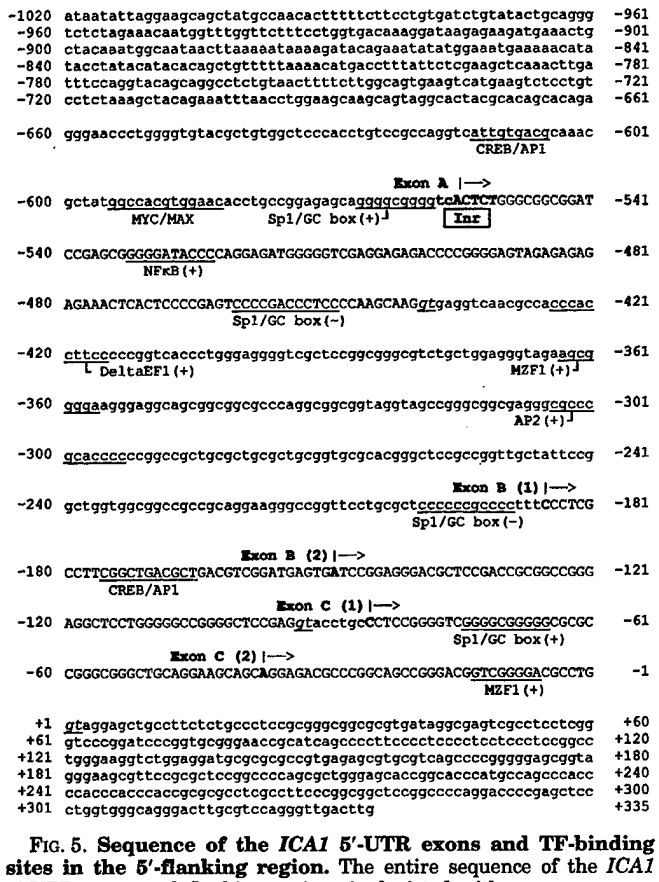


FIG. 5. **Sequence of the *ICA1* 5′-UTR exons and TF-binding sites in the 5′-flanking region.** The entire sequence of the *ICA1* 5′-UTR exons and flanking regions is depicted with exon sequences displayed in *capital letters* and intron sequences in all *lowercase letters*. The consensus Inr sequence at the major initiation site for exon A is in *boldface*, as are the major initiating nucleotides for exons B and C. Although the exon A Inr is an ideal match to the pyrimidine (Py)-rich consensus Inr sequence ($PyPyA^{+1}N(T/A)PyPy$), the initiating nucleotides for exon B transcription also exist within pyrimidine-rich tracts that associate closely with Sp1-binding site motifs. Other close matches to TF consensus sequences are indicated as *underlined segments*. The strand polarity of TF recognition sequences is indicated by either a + for sense or − for antisense orientation.

on evidence for cellular ICA69 expression assessed by RT-PCR and on their similarities with tissues having the highest levels of ICA69 expression, namely pancreatic islets (rat insulinoma cells, RIN 1046-38) and neuronal tissue (human neuroblastoma cells, NMB7).

The results of our *ICA1*-luciferase promoter reporter assays are summarized in Fig. 6*C*. Transfection of construct ExABC −1012 resulted in 1.3- and 3.9-fold increases in reporter gene (luciferase enzyme) activity in RIN 1046-38 and NMB7 cells, respectively, as measured in a dual luciferase assay. Interestingly, however, transfection of cells with the independent exon A and B constructs (ExA −957 and ExB −440) demonstrated a difference in promoting activity that was dependent upon the transfected cell type. The ExA −957 construct was more active in RIN 1046-38 (rat insulinoma) cells, exhibiting a 2.5-fold increase in luciferase activity, whereas the ExB −440 construct showed a greatly enhanced, 12-fold level of activity in NMB7 (neuroblastoma) cells. The augmentation of luciferase activity for the isolated exon A and B constructs over the ExABC −1012 construct is thought to result from the exclusion of splice donors from the exon 3′ ends that may be facilitating cryptic splicing in the luciferase transfection system. The construct designed to isolate exon C from the other 5′-UTR exons (ExC −90), includes the exon C splice donor signal and 19 bp of 3′-flanking sequence. Transfection of this plasmid did not result in any significant increase in luciferase activity as compared with the empty pGL3 Basic control vector in either of the cell lines tested.

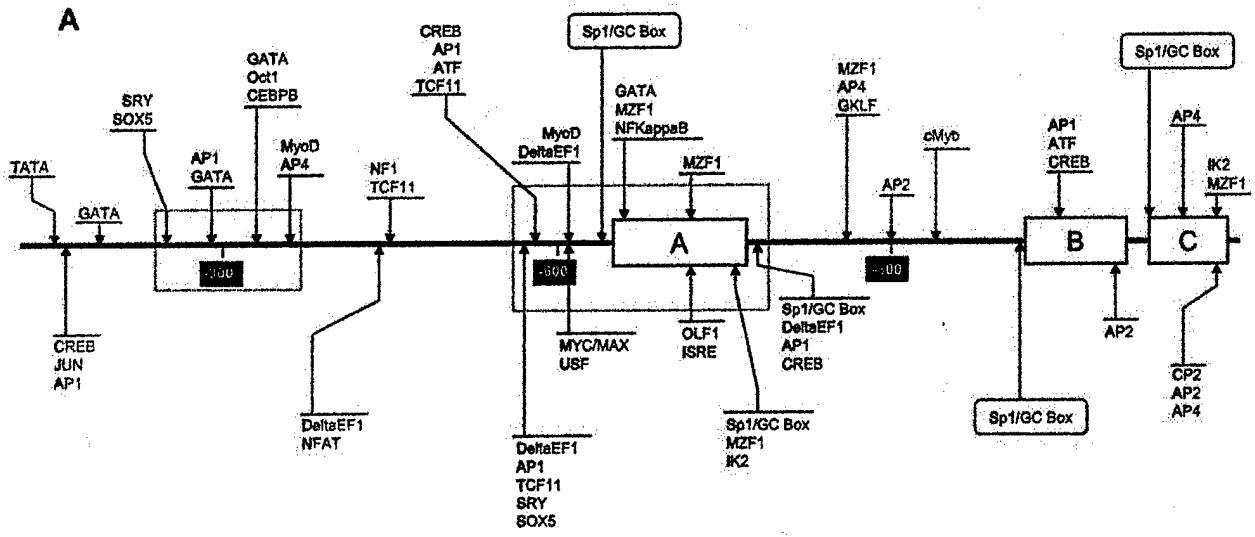
Functional Impact of Site-directed Mutagenesis of ICA1 Promoter Elements—The results of luciferase assays involving the mutated ExA −957 (Fig. 7*A*) and ExB −440 (Fig. 7*C*) constructs are summarized in Fig. 7, *B* and *D*. In NMB7 cells, it is again obvious that there is very little transcriptional activity from exon A, as the parent ExA −957 construct and all four mutants showed essentially no activity over background pGL3 Basic transcription (Fig. 7*B*). This finding correlates with the results of our transcript analysis detailed above. For RIN 1046-38 cells, however, the ExA −957 mutants exhibit increases in promoter activity for mutation of the Sp1/GC box and the Inr independently, as well as for the double mutant combination of the two (Fig. 7*B*). The ExA-CREB mutant shows no difference in promoting activity over the parent ExA −957 construct (Fig. 7*B*).

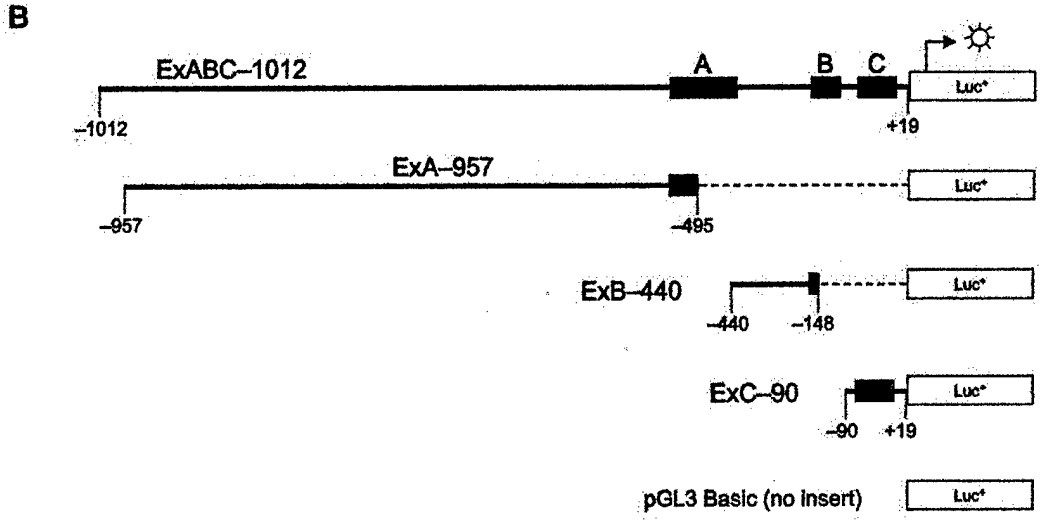
For exon B mutants, there are some very dynamic changes seen in both cell lines, particularly with mutation of the CREB site (Fig. 7*D*). There is a 6.3-fold increase in luciferase activity in RIN 1046-38 cells when transfected with the ExB-GC mutant, whereas a similar increase is not seen when ExB-GC is transfected into NMB7 cells (Fig. 7*D*). Interestingly, however, mutation of the exon B CREB site results in a marked augmentation of luciferase expression in both RIN 1046-38 and NMB7 cells, with 14.7- and 22.9-fold increases in luciferase activity over pGL3 Basic, respectively, corresponding to 7.7- ($p < 0.05$) and 2.0-fold increases in activity over the parent ExB −440 vector (Fig. 7*D*).

DISCUSSION

In the present study, we have explored the complex structure and functional characteristics of the diabetes-related autoantigen gene *ICA1*. Initiation of *ICA1* transcription is found to originate from any of three distinct 5′-untranslated exons having independent transcription initiation signals characteristic of non-TATA, non-CAAT, GC-rich promoters. Transcripts utilizing each of the three 5′-UTR exons coexist in many *ICA1*-expressing tissues, although the 5′-UTR exon sequences are never included together in the same transcript. We present evidence, however, that exon A transcripts predominate in islets and testis RNA samples as compared with exon B or C transcripts, whereas exon B transcripts are the major expressed form in neuronal and cardiac tissue RNA samples. No additional *ICA1* 5′-UTR sequences were detected. An earlier report (4) had suggested that the three 5′-UTR exons are alternatively spliced, but our data conclusively demonstrate that the identified 5′ termini of these exons lack the appropriate splice acceptor consensus sequences (Fig. 5). Furthermore, the 5′-RLM-RACE procedure identified the same 5′ end initiating nucleotides among *ICA1* transcripts amplified from different tissues, suggesting that these initiation sites are common starting points for transcription rather than artifacts of the amplification procedure.

Parallel functional studies to screen cloned *ICA1* 5′-flanking sequences for transcription promoting activity further support the conclusion that utilization of *ICA1* 5′-UTR exons for transcription initiation is tissue-specific. Specifically, a promoter reporter construct containing only exon A and its upstream flanking sequence showed greater activity in islet-derived RIN 1046-38 cells than in neuroblastoma cells, whereas a second construct designed from exon B and its upstream flanking sequence was preferentially active in the neuron-derived cells rather than islets. Based on sequence surrounding the identified transcription initiation sites, we originally hypothesized that the Sp1/GC box-Inr element paring was likely to play a





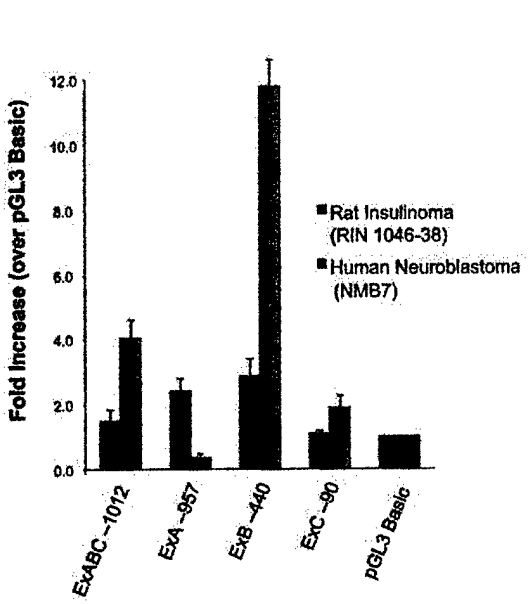


FIG. 6. *A*, TF-binding site sequence matches identified by the MatInspector version 2.2 program are illustrated in a schematic depiction of the *ICA1* 5′-UTR exons and flanking region. Clustering of potential TF-binding sites surrounding exon A and around −900 is indicated by *dotted outlines* on the figure. Each of the three exons is preceded by an Sp1/GC box site, whereas exon A has two additional Sp1/GC box sites at its 3′ end. Of the remaining TF indicated on the figure, many are constitutively expressed in cells, but others are noted to be somewhat more tissue-specific (*i.e.* MyoD and OLF1). *B*, summary of luciferase reporter vector constructs used to assess *ICA1* 5′-flanking region promoter activity. Shown are the

role in the activation of transcription from exon A, given the common association of these consensus sequences reported in the literature (18). However, when promoter sequences were mutated at these sites, there was an associated increase in the activity of luciferase transcription, at least as measured in islet-derived RIN 1046-38 cells. Although these experiments do not provide definitive evidence to suggest a precise molecular mechanism to account for this observation, they imply that the Sp1/GC box site plays an inhibitory role in controlling exon A transcription. It seems reasonable to postulate that Sp3, the Sp transcription factor family member associated with transcription inhibition (19–21), rather than Sp1, is the major transcription factor recognizing and binding to the GC box site in RIN 1046-38 cells. With mutation of the exon A GC box, Sp3 cannot bind as well to the sequence and, therefore, has less chance to inhibit expression from exon A. Similarly, a modest increase in transcriptional activity within RIN 1046-38 cells is noted when the exon B Sp1/GC box site is mutated, perhaps additional evidence that Sp3 plays a role in controlling *ICA1* transcription within RIN 1046-38 cells, in contrast to NMB7 (neuronal) cells where these mutations have no effect. Further experimentation, such as electrophoretic mobility shift assays, designed to explore the potential for Sp1/3 binding to the GC box sites will be necessary to provide support for these hypotheses (22).

An understanding of the role for CREB site binding proteins in controlling transcription from *ICA1* exon B will also benefit from additional studies of transcription factor binding to *ICA1* promoter sequences. Mutation analysis of the exon B CREB site, resulting in marked increases in luciferase activity for both RIN 1046-38 and NMB7 cells, suggests a negative regulatory role for CREB on gene expression through binding to regulatory elements in the *ICA1* promoter (23–26). Likewise, Reusch *et al.* (23, 27) reported that CREB plays a pivotal role in adipocyte survival likely regulating the expression of specific pro- and anti-apoptotic genes such as Akt. Thus, our data suggests that this CREB-related influence may be less cell type-specific than the effects of mutation at the GC box sites, but the effect on expression in pancreatic islet-derived cells does appear to be of a greater magnitude than that noted for neuron-derived cells.

The transcription factor CREB and its wide profile of inducibility has mainly been implicated in glucose homeostasis, growth factor-dependent cell survival, and T-cell receptor signaling (28). CREB was the first transcription factor for which it was demonstrated that phosphorylation regulates its activity; the molecule is activated by cAMP and a variety of other signals. Its family members consist of the activating transcription factor 1 (ATF1) and the cAMP-response element modulator. CREB is a substrate for a host of cellular kinases including AKT (29), p38/Ras (30), MAP-KAP-2 (31), protein kinase C (32), $pp90^{rsk}$ (33), and calcium-calmodulin kinases II (34) and IV (35). Although CREB is perhaps one of the most studied phosphorylation-dependent transcription factors, relatively little is known about the physiological role of this protein in different cellular microenvironments. There is still discussion on how signal discrimination is achieved within the CREB system. Even though several signals have been shown to promote phosphorylation of CREB at Ser-133, it is assumed that CREB can distinguish cAMP from non-cAMP signals at the level of coactivator CREB-binding protein recruitment (28). The characterization of cofactors modulating CREB-binding protein commitment to a specific signaling pathway from a wide array of cellular stimuli is currently under investigation.

Knowing that mutation at the CREB site within the *ICA1* exon B promoter enhances *ICA1* transcriptional activity will be of importance in completing ongoing experiments to augment expression of ICA69 in islet cell lines that, in turn, may aid in elucidating the role for ICA69 in trafficking between the trans-Golgi network and immature secretory granules of pancreatic β-cells that has been proposed (36).

Two additional elements of *ICA1* promoter structure are likely to provide important clues toward understanding the tissue-specific aspects of *ICA1* promoter function. First, the high density of potential TF-binding sites surrounding the three UTR exons provides fodder for mechanisms of transcription activation or repression based on TF availability in different tissues and on the potential involvement of known tissue-specific factors with potential binding sites in the *ICA1* promoter region, like GKLF (gastrointestinal tract), OLF1 (olfactory neuroepithelium), MyoD (myogenic cells), or MZF1 (myeloid cells). Second, existence of the *ICA1* promoter within a genomic CpG island hints at a role for DNA methylation/demethylation in the control of *ICA1* expression. The methylation state of genomic DNA, most often within CpG islands, plays a central role in the epigenetics of gene imprinting (38–41) and has been implicated, although perhaps not proven (37), as an on/off switch for cell type-specific gene expression during cellular differentiation (41–47). The observed differences in *ICA1* promoter activity between RIN 1046-38 and NMB7 cells will be better understood from studies investigating TF recognition and modulation of the *ICA1* promoter and through an analysis of DNA methylation within native genomic DNA of the *ICA1* CpG island sequence.

The variability of *ICA1* transcripts, with respect to both the identity of 5′-UTR sequences and coding region splicing events, is likely to impact ICA69 protein expression. The potential contribution of alternative *ICA1* splice forms to ICA69 translation is obvious (4), although no studies to date have provided definitive evidence for the existence of different ICA69 protein isoforms. On the other hand, 5′-UTR sequence identity could influence *in vivo* ICA69 mRNA stability and translation efficiency, depending upon the secondary structure of the 5′-UTR (48) and upon the number of ATGs contributed by a given 5′-UTR upstream of the accepted ICA69 translation start site in exon 1 (49). Although some variability of the exon 1 splice acceptor for 5′-UTRs has been detected, all human *ICA1* transcripts known to us splice the 5′-UTR to one of a few closely related exon 1 consensus acceptors upstream of the translation initiation codon (Ref. 4 and data not shown). Therefore, it is unlikely that truly alternative translation initiation can occur. Identifying which of these factors further enhances the tissue- and cell type-specific differences in *ICA1* expression that we have observed and to what extent they influence ICA69 protein translation remain important unanswered questions in our

basic structures of the four luciferase reporter vectors constructed using portions of *ICA1* flanking region sequence and 5′-UTR exons. *Dashed lines* indicate where sequence is missing, such that the joined segments would be directly juxtaposed in the plasmid vector. The ExA −957 and ExB −440 were specifically designed to omit the native exon A and B splice donors, respectively, so that cryptic splicing events would be less likely to affect luciferase gene translation. *C*, results of luciferase reporter assays for *ICA1* promoter constructs. Each of the four experimental constructs and the control (no insert) vector were transfected separately into each of the two cell lines indicated. Fold increase in luciferase activity was calculated from the raw data set as described in the text. Transfection of the exon A and B only constructs (ExA −957 and ExB −440) resulted in opposite expression profiles in the islet- *versus* neuron-derived cell lines. This observation is consistent with a tissue-specific pattern of expression from the *ICA1* exons A and B. Expression from the exon C construct was less efficient, perhaps due to the inclusion of the native exon C splice donor in the luciferase reporter vector. Results are expressed as mean ± S.E. of 3–5 independent sets of transfection experiments performed in triplicate.

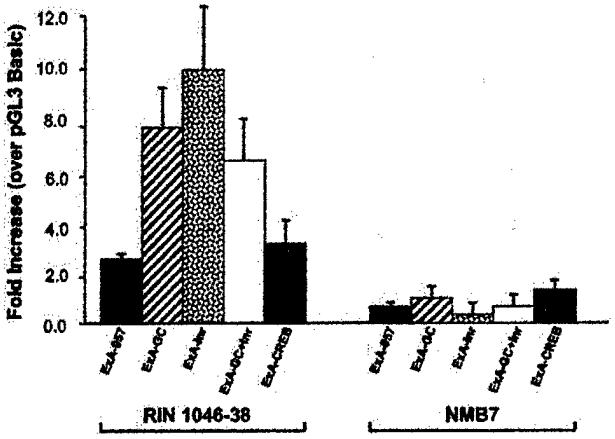
A

```
WT   (ExA-GC,-564 to -561)    GGGGCGGGGTC
Mut  (ExA-GC,-564 to -561)    GGGTATTGGTC

WT   (ExA-Inr,-557 to -553)   GTCACTCTGGG
Mut  (ExA-Inr,-557 to -553)   GTGGAGGTGGG

WT   (ExA-CREB,-612 to -609)  CATTGTGACGC
Mut  (ExA-CREB,-612 to -609)  CATCGGCACGC
```

B



C

```
WT   (ExB-GC, -196 to -193)   CCCCCGCCCCT
Mut  (ExB-GC, -196 to -193)   CCCAATACCCT

WT   (ExB-CREB, -174 to -171) TCGGCTGACGC
Mut  (ExB-CREB, -174 to -171) TCGATCCACGC
```

D

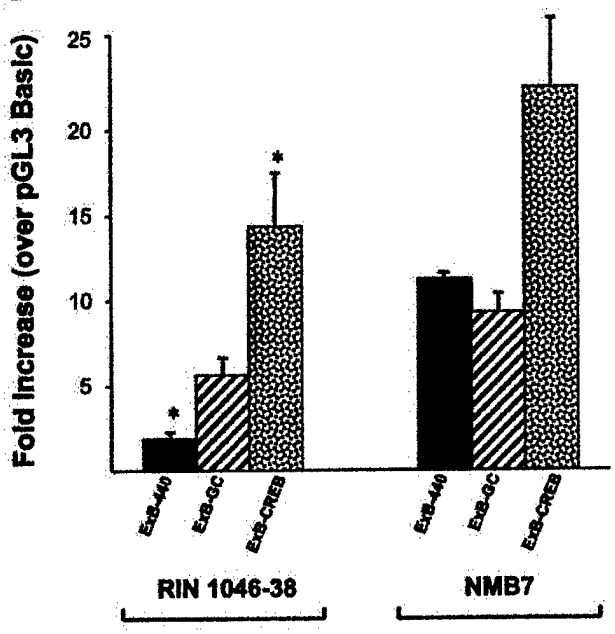


FIG. 7. **Site-directed mutations of promoter elements in transiently transfected RIN 1046-38 and NMB7 cells.** *A*, alignment of the parent exon A (*WT*) and mutant sequences (*Mut*) introduced to the Sp1/GC box (ExA-GC, −564 to −561), Inr (ExA-Inr, −557 to −553), and CREB sites within exon A (ExA-CREB, −612 to −609). *B*, for RIN 1046-38 cells, the ExA −957 mutants exhibit increases in promoter activity for mutation of the Sp1/GC box and the Inr independently, as well as for the double mutant combination of the two [Sp1/GC box + Inr]. The ExA-CREB mutant shows no difference in promoting activity over the parent ExA −957 construct. *C*, alignment of the parent exon B (*WT*) and mutant sequences (*Mut*) introduced to the Sp1/GC box (ExB-GC, −196 to −193), and CREB sites within exon B (ExB-CREB −174 to −171). *D*, mutation of the exon B CREB site leads to a significant increase in luciferase expression in both RIN 1046-38 and NMB7 cells, corresponding to 7.7- ($p < 0.05$) and 2.0-fold enhancement in activity over the parent ExB −440 vector. Results are expressed as mean ± S.E. of at least 3 independent sets of transfection experiments performed in triplicate. By using Mann-Whitney test, *, $p < 0.05$ comparing the mean of results for the mutation of the exon B CREB site with the mean of results for the parent exon B. p values < 0.05 were deemed statistically significant.

quest to understand the biology of this molecule.

The sum of available *ICA1* and ICA69 structural and functional genetic data point to a complex process of transcript generation from an expansive gene locus. Of note, the large introns (largest almost 60 kb) and overall gene size (>150 kb) by nature require a high fidelity of RNA processing to command the expression of a comparatively small protein (483 amino acids). From the standpoint of understanding basic biology, the significance of introns and intron size continues to be a subject of discussion in the literature (50–52). Available cDNA sequences suggest that opportunities for alternative intron/exon processing of *ICA1* transcripts may contribute to isoform variation of ICA69 protein expression (53), although the particular influence that extremely large introns would contribute to this process is unknown. That these large introns contain other genes or genetic regulatory elements is a strong possibility (41, 51), especially considering that two *ICA1* introns measure ~26 and ~60 kb, larger than many whole genes and easily large enough to envelop genetic regulatory elements of importance to *ICA1* gene function. More simply, considering the remarkable cross-species conservation of the ICA69 coding region, perhaps large introns merely confer a relative level of protection against coding region mutations and disruptive recombination events because these processes would be more likely to affect non-coding intron sequences on a statistical basis (54, 55). It is difficult to draw any firm conclusions regarding the significance of such huge introns to the *ICA1* gene; we anticipate that additional collective gene structure data from the various ongoing genome projects will enhance our understanding of these observations.

The basic structure and function of the *ICA1* core promoter units contributed by this work and the suggestion of candidate regions harboring additional *ICA1* 5′-regulatory sequences will facilitate the targeted screening of genomic regions for polymorphisms that could alter *ICA1* promoter function. There is already evidence that a polymorphic variable number of tandem repeats in the insulin promoter is functionally important to variations of insulin expression and correlates with diabetes susceptibility (56–60). In addition, multiple research groups have recently reported the expression of ICA69, insulin, and the other major diabetes autoantigens glutamic acid decarboxylase (GAD65) and IA-2 within cells of the thymus (60–65) and in peripheral lymphoid organs (66). The centrality of these lymphoid tissues to the maintenance of immune self- tolerance suggests that perhaps at least one determinant of which proteins are targeted as autoantigens is the level or nature of expression in tissues other than where it has its characteristic biological effect. Immunohistochemical and molecular analyses of autoantigen-containing cells within lymphoid tissues have identified features of a dendritic cell phenotype (65–67), implicating these powerful antigen presenting cells in the process of establishing and maintaining immune tolerance via *de novo*

expression of self-protein antigens. These observations lead us to postulate that inheritance of functional polymorphisms within promoters controlling the expression of identified autoantigens will ultimately be correlated with the occurrence of autoimmunity and autoimmune diseases. With our fundamental understanding of the structure and function of the *ICA1* gene promoters in hand, a rational approach to identify relevant *ICA1* promoter polymorphisms and to investigate disease associations is now feasible.

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REVIEW

Gene- and cell-based therapeutics for type I diabetes mellitus

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Type 1 diabetes mellitus, an autoimmune disorder is an attractive candidate for gene and cell-based therapy. From the use of gene-engineered immune cells to induce hyporesponsiveness to autoantigens to islet and beta cell surrogate transplants expressing immunoregulatory genes to provide a local pocket of immune privilege, these strategies have demonstrated proof of concept to the point where translational studies can be initiated. Nonetheless, along with the proof of concept, a number of important issues have been raised by the choice of vector and expression system as well as the point of intervention; prophylactic or therapeutic. An assessment of the current state of the science and potential leads to the conclusion that some strategies are ready for safety trials while others require varying degrees of technical and conceptual refinement.
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Introduction

Of all the autoimmune diseases, none has been as popular a target of cell and gene-based prophylactic and therapeutic interventions as type I diabetes mellitus. The delineation of the cellular effectors and the molecular pathways involved in the breakdown of central and peripheral tolerance has promoted interventions as diverse as bone marrow transplantation with or without antibody-based immunosuppression for tolerance induction, transplantation of genetically engineered islets of Langerhans to restore insulin production, embryonic and pancreatic stem cells and non pancreatic progenitors as surrogate β cells. Almost all of these strategies have been realized in the non obese diabetic (NOD) mouse model, the workhorse of this area of investigation and, in parallel, yet to a smaller degree pursued in the diabetes-prone BioBreeding (DP-BB) rat. It is worth noting that many of these strategies have successfully prevented diabetes with varying effects on the degree of insulitis (the cellular inflammation in and around the islets) as outlined by Atkinson and Leiter.[1] in a very notable list. In humans, to date, the only clinically acceptable treatment for type I diabetes, other than insulin replacement, remains islet transplantation under the cover of pharmacologic immunosuppression. A very recent safety trial has begun using an anti-CD3 antibody, but the results require confirmation and further safety analysis. Whether the cell- and gene-based strategies published in the last decade will ever be clinically translatable is not yet clear. We undertook to review these strategies and their potential clinical utility with the ultimate objective of giving a perspective on the field from the view of gene therapy.

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Type I diabetes mellitus: the autoimmune process

There is no doubt that type I diabetes mellitus (TIDM) is genetically determined and is triggered by an as-yet unidentified postnatal determinant, very likely environmental in nature. The genetics of the disease is multifactorial and involve two loci (*IDDM1* and *IDDM2*) confirmed to be in linkage with the disease. *IDDM1* encompasses the HLA gene complex and it alone defines the most important risk factor. In humans, the disease is associated with the inheritance of DR3/DR4 haplotypes (DR3: DQA1*0501, DQB1*0201 and DR4: DQA1*0301, DQB1*0302).[2,3] *IDDM2* has been mapped to a variable number of tandem repeats (VNTR) polymorphism upstream of the insulin gene promoter, which can determine thymic levels of insulin.[4,5] In fact, a recent study demonstrated that the number of active copies of insulin in a transgenic mouse can influence the degree of immune cell reactivity towards insulin, a putative autoantigen.[6] A number of other loci have demonstrated suggestive associations, but to date, none of these results have been replicated to establish significant linkage with the disease.[7–9]

A number of earlier hypotheses with some supporting evidence have been put forward to explain the possible mechanism of action of the environmental trigger including β-cell death secondary to virally triggered inflammation, molecular mimicry, superantigens and diet.[10–19] What is certain is that at some point postnatally, the immune system of a genetically predisposed individual is activated to infiltrate chronically the islets of Langerhans. While the initial phase of infiltration may not involve β-cell destruction, a number of studies *in vivo* and *in vitro* suggest that immune cells become able to render β-cells dysfunctional through the actions of cytokines they produce such as interleukin-1β.[20–23]

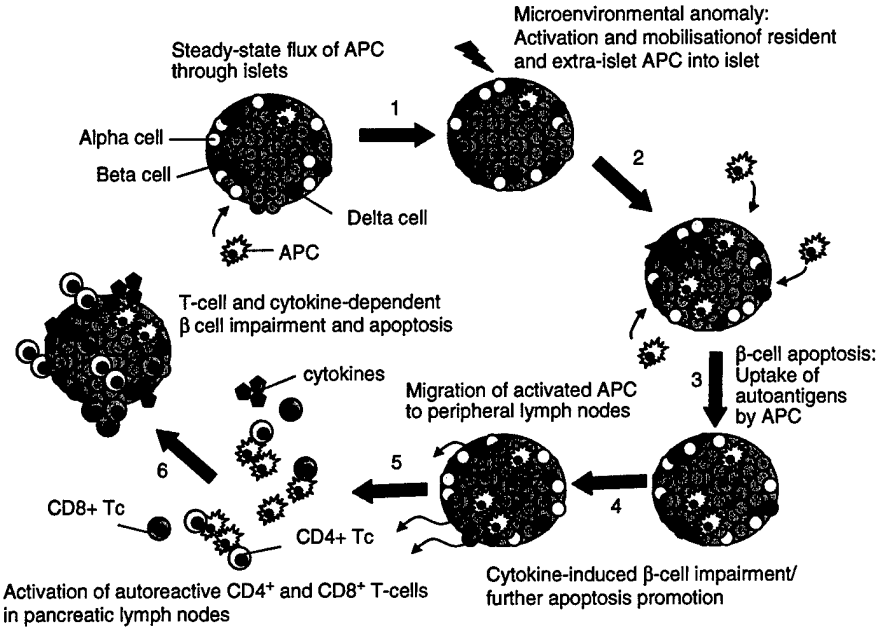


Figure 1 Multistep process of insulitis. During ontogeny, a population of thymocytes whose TCR recognize β-cell-specific antigens are either not deleted in the thymus, or fail to be tolerized subsequently, in the periphery. These T cells may circulate dormant, or may be active, but suppressed by regulatory T-cell networks. Islet-resident APC (DC or macrophages) are normally in a steady-state flux sampling the microenvironment. An as-yet unidentified microenvironmental anomaly shifts their phenotype into activators of an inflammatory response, as they migrate out of the islet environment and into the peripheral lymphoid organs. There, they eventually encounter the autoreactive T cells. In the meantime, antigen nonspecific inflammation progresses within islets because of macrophage and DC secretion of soluble mediators of β-cell dysfunction and apoptosis activation.

Figure 1 illustrates what many believe to be the course of islet inflammation. Many lines of evidence indicate that antigen-presenting cells (APC), especially dendritic cells (DC) are pathologically activated to orchestrate the insulitic process.[24] It is thought that islet-resident APC respond to a microenvironmental anomaly (perhaps β-cell death and/or impaired β-cell turnover or apoptosis[25–27]) and initiate the insulitis process by migrating out of the islets and into the peripheral pancreatic lymph nodes. By presenting the β-cell antigens they have acquired, the APC interact with β-cell-reactive T cells, which escaped thymic deletion and trigger their activation and proliferation (Figure 1). Once activated, these T cells act as one pole of a chronic tug-of-war between β-cell-specific autoimmunity and peripheral mechanisms of tissue-specific tolerance. Regulatory T cells of various cell surface phenotypes and cytokine secretion profiles may also be involved in modulating this unstable equilibrium. Ultimately, this chronic process ends in favor of the β-cell-reactive T cells, which eventually end up destroying enough β-cell mass to render the patient insulin-dependent. This process has been extensively studied in the NOD mouse and is believed to occur in humans as well.

Lessons to be learned: gene vectors or cells or both?

An appreciable amount of work has focused on using viral vectors to infect intact islets in culture prior to transplantation into recipients to impede the allogeneic rejection (reviewed in Giannoukakis *et al*[28,29]). The excitement generated by these studies, however, was tempered by the appreciation that permanent allograft survival was generally not achieved. Often, to explain this limited success, investigators invoke the immunogenicity of the particular vector used, although recent evidence suggests that the quality of the islets may be more crucial than the vector choice in determining the presence and grade of inflammation in and around the graft.[30] Table 1aa lists the vectors that have been used to date to transduce intact human islets as well as their pros and cons (Table 1c). (Table 1b lists their properties). The list indirectly demonstrates that no 'ideal' vector yet exists. New technology, including small interference RNA (siRNA),[31] adeno-associated virus inverted terminal repeat (AAV ITR)-based plasmids,[32,33] novel classes of lentivirus (equine infectious anemia virus-EIAV; feline immunodeficiency virus- FIV),[34–40] lentivirus–herpesvirus hybrids and other viral vectors is in development, but their efficiency has yet to be reported in the context of

Table 1a Gene vectors that transduce islets (with references)

Plasmid DNA[202–205]
Adenovirus[206–219]
Adeno-associated virus[114,115,130,132,220–228]
MoLV retrovirus[229]
Lentivirus[230–232]
Herpes simplex virus[233,234]
Cationic liposomes[204,205,214]
Peptide fusion domains[118,235]

Table 1b Properties of gene transfer vectors with applicability to islet gene transfer

Vector type	*Stable transduction*	*Cell cycle requirements*	*Immunogenicity*	*Islets transduced?*
Plasmid DNA	No	Dividing/nondividing	Yes	Mouse/human
Adenovirus	No	Dividing/nondividing	Yes	Mouse/human
Adeno-associated virus	Possibly	Dividing/nondividing	Minor	Human
MoLV-based retrovirus	Yes	Dividing	No	Mouse/human Very poor
Lentivirus	Yes	Dividing/nondividing	No	Mouse/human
Herpes simplex type-1 virus	No	Dividing/nondividing	Inherent toxicity	Human
Cationic liposome	No	Dividing/nondividing	No	Human
Peptide fusion domains	No	Dividing/nondividing	No	Human

Table 1c General characteristics of gene delivery vehicles

Vector type	*Pros*	*Cons*
Plasmid DNA	Easy to engineer, grow and purify; multicistronic variants easy to engineer	Poor persistence, nonspecific cell targeting, poor dissue diffusion
Adenovirus	Choice vector for pilot proof-of-principle experiments; High titers easily obtained; almost all cells and tissues are transducible; cell retargeting is possible	Immunogenic in vivo; nonstable transduction
Adeno-associated virus	Site-specific, stable integration achievable, almost absent immunogenicity; many cell types transducible	Time for transgene expression can be on the order of days
MoLV-based retrovirus	Stably integrating vector in rapidly dividing cells; cell-type retargeting possible; good titers obtainable	Subject to chromosomal position-dependent suppression, as well as methylation and cytokine effects on gene expression;
Lentivirus	Nonimmunogenic, stably integrating; choice vector for nondividing, noncycling cells; good titers obtainable; Data support absence of replication-competent-recombinant vector particles in stocks	Clinical safety concerns with HIV-1-based vectors
Herpes simplex type-1 virus	Large genome available for multiple large size cistrons; good persistence in many cell types; cell-type retargeting possible	Inherent toxicity
Cationic liposome	Easy to manipulate to deliver plasmid DNA to almost all cells and tissue. Nonimmunogenic; cell-type nonspecific, cell-type retargeting possible	Poor control of diffusion kinetics
Peptide fusion domains	Many cell-types transducible; high-level proein/peptide import; intact proteins/peptides delivered; not subject to gene regulation; targeting of specific proteins possible; high-level peptide production easily achievable; no reported immunogenicity	Short half-life; subject to proteolytic degradation; large amounts require some time to generate

intact islet transduction. Equally unknown is the degree to which these vectors can contribute to post-transplantation inflammation.

Cell therapy constitutes an alternative approach to induce tolerance to alloantigens. Allogeneic bone marrow transplantation, with or without the addition of immunoregulatory antibodies (blocking CD28:B7 and CD40:CD40 ligand interactions), has been the choice of many investigators to promote allogeneic islet transplantation in mouse models of autoimmune diabetes.[41–50] In some instances, permanent allograft survival has been reported in prediabetic mice (permanent in the sense that the recipient maintained normoglycemia at the time it was last tested). It is not clear, however, if these strategies would work equally well in an already-diabetic individual. A number of studies attempted to promote the activity of regulatory immune cells by DC. This novel and rational approach, however, may require multiple administrations to maintain a sufficient level of activity.[51,52] Combinations of these approaches, including gene-engineered DC expressing a variety of immunosuppressive molecules, have shown promise in allograft survival.[53–59] and are awaiting rigorous testing in the context of islet allograft transplantation. Considering successes and failures, it is perhaps fair to conclude that while gene vectors and cells alone may not have yet supported permanent islet allograft survival, their utility cannot be yet dismissed as many important parameters have still to be evaluated, including combinative approaches. In fact, very few studies have attempted to engineer islets expressing more than one immunoregulatory transgene at a time. This is an important aspect of the problem to consider since the immune response against the transplant (and perhaps the vector) may involve more than one pathway.

A General overview of strategies: prevention versus insulin replacement

Other than insulin replacement by daily injections of the hormone, the only other clinically acceptable means of insulin restoration remains islet transplantation.[60–63] Recent advances in understanding transplantation immunology in general and the process of insulitis and the molecular/genetic bases of failure of central and/or

peripheral mechanisms of tolerance to tissue-restricted antigens, in particular, have yielded a number of approaches for therapy and prevention of TIDM. To manipulate the immune system in a prophylactic manner, cell- and gene-based modalities or a combination of both were tested. Therapeutic strategies strive instead to improve islet transplantation by improving insulin secretion, engraftment and most importantly, protection of the transplant from allogeneic immune rejection. In humans, islet grafts derive from allogeneic cadaveric donors. Stem/progenitor cells, alone or in engineered form, are also potential candidates for β-cell replacement. Each approach has shown promise, but each approach has also demonstrated its limitations. New data suggest that a critical period between time of diagnosis and actual destruction of β-cell mass required for appropriate glycemic control (the so-called 'honeymoon period'; see below) may be exploited immunologically to obviate the need of islet transplantation altogether. While antibody-based approaches are currently being tested, it is anticipated that emerging gene and cell therapies can overcome the safety and negative systemic effects associated with the antibody approach.

Prevention strategies

In order to prevent the disorder, one must be able to identify first with a sufficient degree of confidence individuals who are at very high risk for developing type I diabetes. While inheritance of susceptibility alleles at loci linked to and/or associated with the disorder is an important risk factor, it alone cannot guarantee that the individual will in fact become diabetic. This is the main reason for the ongoing debates on prevention based on genetic screening.[2,64] While outright prevention based only on genetic screening may not be yet acceptable, other strategies that fall inside the realm of 'prevention' can be acceptable. There are data indicating that newly onset diabetics still possess adequate β-cell mass to sustain normoglycemia if the autoimmune inflammation can be promptly controlled.[65–71] The time between diagnosis and elimination of β-cell mass adequate to sustain normoglycemia has been termed the 'honeymoon' period. One can exploit immunoregulatory networks to promote hyporesponsiveness of autoaggressive immune cells in this period as a viable means of improving or restoring normoglycemia. Supporting this approach are the studies where treatment of newly onset diabetic NOD mice with an anti-CD3 antibody restored normoglycemia in a substantial portion of mice for a sustained period of time.[72] Very recently, human trials using the same approach also seem quite promising.[73] Although clinical diabetes onset has most often been associated with β-cell death, it is possible that the low levels of insulin production are because of the effects of cytokines that modulate their production. If this is the case, this process can be reversed.[21–24,74–76] Some data strongly suggest that suppression of the activity of the insulitic cells by the induction of immune hyporesponsiveness in clinically diabetic individuals may promote either β-cell neogenesis and/or rescue of the cytokine-suppressed β cells in the insulitic environment.[50,77,78]

Inherent in this philosophy is the ability to promote TIDM-specific autoantigen tolerance or TIDM-specific autoantigen immune hyporesponsiveness. To acheieve this, one can target genes and/or cells to the thymus, or one can manipulate the peripheral immune effectors using cells alone or gene-engineered cells. These approaches are illustrated in Figure 2. The evidence suggesting that a preventive approach manipulating the thymic environment of antigen presentation is possible was initially obtained by generating transgenic NOD mice with different H2 (major histocompatibility complex) genes. Mice carrying H2 transgenes conferring resistance did not develop diabetes.[79–83] Additionally, diabetes in the NOD mouse was also prevented by thymic inoculation of soluble islet antigens in the form of cellular lysates or by expression of putative β-cell autoantigens in the thymus.[79,84] Could this approach be clinically applicable? Recent data on plasticity of bone marrow stem cells[85–88] seem to imply that culture conditions could be defined in which bone marrow progenitors could be propagated towards 'thymic' APC. These cells could be engineered using a number of viral or nonviral vector methods (gene vectors to be described in a later section) to present autoantigen. These cells could then be injected into the host where they could eventually populate the recipient thymus. To obviate the problems associated with graft *versus* host disease in an allogeneic context, one could envisage the use of hematopoietic stem cells propagated from peripheral blood precursors of the recipient. Preliminary evidence seems to suggest that the newly generated insulin-generating cells may not have the same phenotypic makeup of normal β cells and because of this characteristic, they may be able to escape the recurrence of pre-existing autoimmunity.

A number of studies have shown that allogeneic bone marrow transplantation into NOD or BB rats with the aim of inducing a state of chimerism can also prevent diabetes and facilitate allo- and xenograft islet transplantation.[41–50,89]. While the mechanisms are believed to involve central and peripheral chimerism, the applicability of this approach in humans is impeded by the use of very high radiation conditioning of the recipient. The need for complete or partial myeloablative treatment and of allogeneic donors could be obviated by genetically engineering peripheral blood-derived autologous hematopoietic stem cells with transgenes promoting the induction and activity of immunoregulatory networks. Independently of the means utilized to abrogate autoimmunity, a state in which the diabetic patient is free of autoreactive T cells and their assault on pancreatic β cells is optimal to allow or promote the rescue or regeneration of enough insulin-secreting cells in the endogenous pancreas. This may allow physiologic euglycemia. Alternative measures to control the glycemia during the possibly long recovery period must also be implemented.

Case for DC

Although considered as potent immunostimulators, DC have recently been shown to possess tolerogenic characteristics under defined conditions. DC tolerogenicity manifested as the suppression of T-cell activation has been documented in tumor, allo- and autoimmunity.[90]

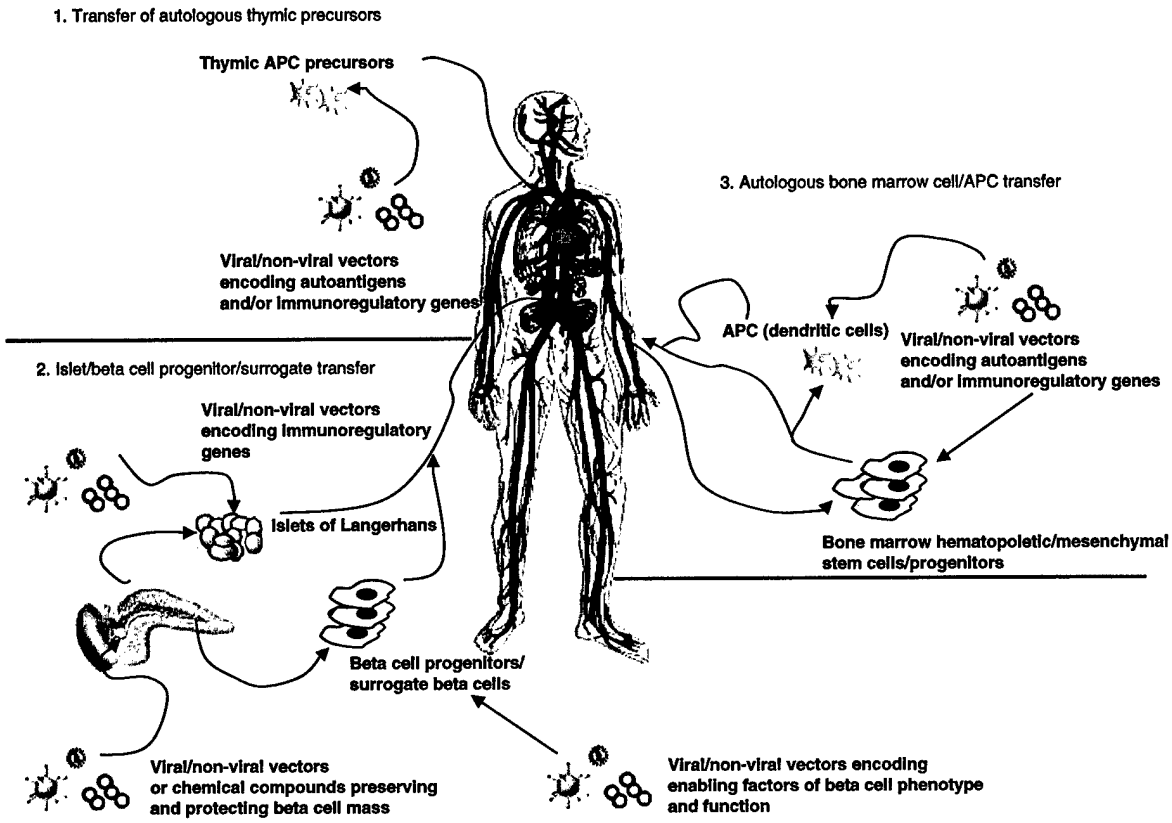


Figure 2 Gene and cell therapy strategies to promote islet allo-/xenograft survival and/or prevent diabetes A number of strategies can be employed alone or in combination: (1) gene transfer of immunoregulatory molecules to autologous cells that can modulate immunoregulatory networks (such as dendritic cells); (2) gene transfer of cytoprotective genes to allo- or xenogeneic islets that will be subsequently transplanted; (3) protection of β-cell mass and function during the isolation phase of islets; (4) gene transfer of factors which promote a β-cell phenotype to β-cell progenitors or surrogate cells; (5) direct transfer of vectors encoding immunoregulatory molecules into susceptible or a recent-onset patient.

The conditions that can yield tolerogenic DC include ultraviolet irradiation, as well as exposure to cytotoxic T-lymphocyte antigen-4:immunoglubulin Fc fusion (CTLA-4Ig), transforming growth factor β (TGF-β) or interleukin (IL)-10.[91–93] How a tolerogenic DC acts to suppress immunoreactivity is not completely understood, but may involve the promotion of anergy of T cells that come into contact with DC, a shift from TH1- to TH2-type responses, apoptosis of the autoreactive T cells or the induction of regulatory cells including regulatory T cells and natural killer-T (NK-T) cells.[90,94–98] With the aim of establishing a durable tolerogenic state in the recipient of an allogeneic transplant, myeloid DC have been genetically modified using adenoviral and retroviral vectors encoding CTLA-4Ig, TGF-β and IL-10 in the mouse.[91–93]. CTLA-4Ig-expressing DC significantly prolong allograft survival, can induce alloantigen-specific T-cell hyporesponsiveness, and display enhanced survival in nonimmunosuppressed, allogeneic hosts.[92] The *in vivo* presentation of alloantigens by donor or recipient DC in the absence of costimulation along with local production of immunosuppressive molecules like TGF-β, could likely promote the inhibition of antidonor reactivity and promote tolerance induction without causing any major systemic immunosuppression. DC engineered to express vIL-10 following retroviral gene transfer produce high levels of vIL-10 *in vitro*, exhibit marked reduction in cell surface MHC and costimulatory molecule expression, decrease T-cell allostimulation and promote the induction of T-cell hyporesponsiveness.[91] Genetically engineered DC may be used to prevent islet allograft rejection, since they are able to manipulate antidonor and/or autoantigen immunoreactivity. If recent observations showing islet-specific molecule gene expression in peripheral lymphoid organs can be confirmed in APC[99] like bone marrow-derived DC (Machen *et al*, unpublished observations), one can envision infusing autologous DC-engineered *ex vivo* to lack costimulatory capability, but also express islet-specific genes (eg, glutamic acid decarboxylase (GAD)-65 or insulin), into prediabetic or early onset diabetic patients, with the objective of inducing autoantigen-specific hyporesponsiveness. In fact, DC have been treated *ex vivo* with oligodeoxyribonucleotide decoys to nuclear factor κB (NF-κB), an important maturational transcriptional mediator in DC, and injected into an allogeneic host. These DC were able to prolong the survival of an allogeneic heart.[100] It is likely that this and other transcriptional pathways in APC could be exploited by decoy nucleotide strategies to present autoantigen in the absence of

costimulatory signals or in the presence of death ligands to silence or kill autoreactive T cells.

Insulin replacement strategies

Novel immunosuppressive cocktails, culture in the presence of homologous serum proteins, minimization of time between pancreas procurement and islet processing combined with transplantation of a larger β-cell mass were the most significant steps in improving islet transplantation outcome in the studies of Shapiro and Co-workers.[101–104] Although it is not clear which of the parameters contributed most to success, many factors still limit a large-scale diffusion of islet and β-cell replacement for type I diabetic patients. The need for chronic immunosuppression and for multiple donors as a source for islets remain the prime reasons or factors that impose a search for alternative ways of promoting islet cell allograft survival. Tolerogenic protocols, once successful, may allow the use of islet transplantation in young diabetic patients.

Gene transfer technology is such an option and a number of advances have been attained in animal models of islet allograft transplantation. Tables 2a and 2b lists experiments in which significant prolongation of islet allograft or xenograft survival has been achieved.

The main obstacle for a gene transfer-based approach is the choice of gene transfer vectors. Despite initial enthusiasm about the versatility of adenoviral vectors, their inherent immunogenicity raises a number of serious concerns in view of their possible application to engineer human islets for clinical use. The advent of lentiviral vectors appeared to alleviate some of the immunogenicity concerns, but lentivirus are not as efficient as adenoviruses in transducing intact human islets. As indicated in an earlier section, Table 1a lists a number of gene transfer vectors as well as their pros and cons in the context of gene transfer to intact islets. However, an underappreciated factor that very likely affects the success of islet engraftment is the metabolic status of the islets themselves following isolation and culture. There is no doubt that the time between organ retrieval and islet processing with the inherent intermediate steps including cold storage and enzymatic/mechanical digestion affects islet yield, viability and function.[30,105] Furthermore, the culture conditions prior to transplantation can crucially affect islet cells physiology and, consequently, the chance of successful engraftment. In general, the cessation of the oxygen supply to the pancreatic tissue at the time of donor organ harvesting is known to trigger ischemic damage, free-radical-mediated cell degeneration as well as initiation of apoptosis.[106,107] Also, the separation of the islets from the surrounding matrix and from the neighbor cells driven by the isolation procedure further contributes to activate cell apoptosis.[108,109]. Immediate-onset ischemia has been proposed to be an important determinant of acute and chronic allograft rejection.[110] In addition, organs carrying contaminating immune and a large number of endothelial cells or in which platelets have been trapped will likely experience a so-called 'cytokines storms', where the onset of apoptototic processes cause an abnormally large release of stored cytokines and other proinflammatory soluble mediators. Moreover, a cycle is initiated whereby cytokines release can exacerbate the formation of reactive oxygen intermediates.[111] Presumably, the combination of all these mechanisms predispose the islets to environmental damage both during culture and at the transplantation site, where inflammation is likely to occur shortly after implant even before alloimmune response starts. Potential approaches to avoid this situation can include the perfusion of the organs with solutions containing chemical inhibitors of apopto-

Table 2a Genes that promote islet allo-/xenograft survival in vitro and in vivo and/or β-cell survival in culture

Anti-apoptotic genes
Bcl-2[114,115,233,234,236]
Bcl-x_L[118,237]
Heme oxygenase-1[112,113,238]
Dominant-negative protein kinase C delta[239]
Dominant-negative MyD88[240]
IGF-I[226]
IκB α super-repressor[227]
Hsp70[241]
A20[216]
PEA-15[118]
Catalase 242,243
Manganese superoxide dismutase[244]

Cytokines
IL-4[245] (although one report demonstrated no protection[206])
Interleukin-1 receptor antagonist protein[228]
IL-12p40[207]
Viral IL-10[208]
IL-10[246] (one report did not show protection[206])
TGF-β[246] (one report showed negative results[247])

Immunoregulatory genes
Indoleamine 2,3-dioxygenase[222]
CTLA-4Ig[203]
Fas ligand[209] (although in a number of reports Fas ligand was not protective:[248])
Adenoviral E3 genes[210]

Table 2b Other gene/cell therapy approaches to prevent/abrogate autoimmunity and/or promote islet allo-/xenograft survival

Bone marrow transplantation/chimerism induction[41,46–49,89,249–254]
Antigen-presenting cell transfer
Class I MHC[255]
Autologous DC transfer,[51]

Costmulation blockade
Soluble ICAM-1-Ig[256]
CTLA-4Ig[257–261]
OX40Ig[260]

Cytokines
IL-10[262–265]
IL-4[263,266]
Soluble IFN-γ receptor[267,268]
TGF-β[269]

Autoantigen transfer
GAD[270]

Others
Adenovirus E3 proteins[271]
Orally administered putative autoantigens (insulin, GAD)[272–274]
CD152[275]

sis (ZVAD-fmk) as well as antiapoptotic genes like bcl-2, bcl-xL, and enzymes that break down, or prevent, the formation of free-radicals such as catalase, thioredoxin, heme-oxigenase-1 and superoxide dismutase.[112–117] Some of these antiapoptotic proteins fused to protein-transduction domains can successfully prevent apoptosis and significantly improve islet yield and survival following isolation.[30,118,119] We and others have also shown that the inclusion of synthetic mimetics of free-radical scavengers seem to prevent islet degeneration possibly limiting the initiation of apoptotic processes.[105,119] Islets also take up oligonucleotides quite efficiently (unpublished observations). Knowledge of the primary transcripts whose protein products are involved in apoptosis activation or suppression of insulin production can be targeted with antisense oligonucleotides during the isolation procedure.

As previously mentioned, oligonucleotide therapy offers a simple and convenient method to interfere with not only gene expression, but also with transcription using short double-stranded decoys containing binding sites for specific transcription factors involved in inflammatory responses, like NF-κB and STATs. Soluble binding proteins and ligand-binding domains of chemokines can also be considered potential tools with which primary islet dysfunction can be prevented. Chemokines are potent immunoattractants fairly resistant to degradation and are sequestered by proteoglycans on the endothelium.[120,121] Chemokines promote endothelial adhesion in addition to their chemotactic properties.[120,121] Virally encoded proteins have been identified that bind chemokines and could be a means of achieving chemokine blockade.[122,123] This blockade can easily be attained using peptide transduction domains fused to recombinant proteins or short oligonucleotides, especially if administered during procurement and reperfusion of the donor pancreas. However, long-term expression of some of these molecules may have a greater effect on graft survival once stable gene expression is achieved. This necessitates the use of gene vectors that can deliver the therapeutic gene with the objective of expression for the entire lifetime of the recipient.

Injection of animals with a number of vectors like adenovirus[124–129] and adeno-associated virus[130–133] encoding proinsulin under the control of a number of promoters including CMV, insulin, phosphoenolpyruvate carboxykinase (PEPCK) and L-pyruvate kinase (LPK) has resulted in correction of hyperglycemia. In many instances, however, the effect appears to have been transient. This approach suffers from the potential immunogenicity of the virus, and in many cases precludes a second dosing because of the generation of neutralizing antibodies. Other issues are related to choice of promoter, which in the instance of LPK demonstrates slow kinetics, although one study with this promoter was able to achieve relatively rapid responses to glucose.[131] Finally, many tissues do not express the necessary proteinases that process proinsulin into the potent bioactive insulin.

Surrogate β cells offer an alternative to intact islet transplantation and direct injection of proinsulin-expressing vectors. A variety of cell types including fibroblasts, muscle, neuroendocrine cells and hepatocytes have been engineered to produce insulin.[134–138] The most notable advances have been made using engineered hepatocytes.[126,128,129] Hepatocytes are particularly attractive because they can easily engraft in the liver, and because they possess identical glucose-sensing molecules as the pancreas (eg, GLUT2, glucokinase (GK)). Furthermore, one can exploit a number of hepatocyte gene promoters, which are sensitive to glucose, in order to engineer insulin transgenes to be glucose concentration-sensitive. Despite a number of promising approaches exploiting a number of glucose-regulated promoters,[126,128,129,131,139–144] much more work is needed to make hepatocytes into fully surrogate β cells. The first feature that a hepatocyte is missing to properly act like a β-cell surrogate is the ability to respond to glucose in a sufficiently rapid fashion, as rapid as that characteristic of β cells. Second, the liver-specific glucose-sensitive promoters have elements that respond to hormonal and metabolic signals that can impede, attenuate or abrogate the desired objective of tight glucose regulation. For example, instances of hyperglucagonemia, which is to be expected in the absence of functional endogenous β cells in diabetics, will most likely attenuate or repress the LPK promoter as well as other promoters such as GK.[129,145,146] Third, glucose-dependent trans-activation of the LPK promoter requires GK-dependent phosphorylation of glucose, an activity that is insulin-dependent.[129] Other promoters have been suggested, such as that of PEPCK, but this promoter is activated by glucagon and inhibited by insulin, which may not result in the desired kinetics of physiological gluco-regulation.[147,148] It is possible that a combination of promoter elements from different glucose-responive hepatic genes may be needed to create an optimal synthetic promoter to drive hepatic insulin expression in a true glucose-sensitive fashion.

In an entirely different approach, tissue-specific promoters have been exploited to engineer cells to express insulin in cells that are not targets of autoimmune destruction. Lipes *et al*[149,150] have expressed insulin in the anterior pituitary gland of NOD mice under the control of the pro-opiomelanocortin promoter. Insulin was expressed, stored into secretory granules and exhibited regulated secretion. Moreover, transplantation of transgenic anterior pituitary tissue to NOD mice was able to restore partially normoglycemia without any signs of immune rejection.[149,150] It was not clear, however, if in these cells insulin secretion was glucose concentration-dependent. More recently, an ingenious approach harnessing intestinal K cells as surrogate glucose-responsive insulin producers was demonstrated. In this approach, transgenic mice expressing human insulin under the control of the gastrointestinal inhibitory peptide (GIP) promoter were generated. These mice expressed and secreted insulin from intestinal K cells in which the GIP promoter is active. Insulin secretion in these mice was glucose-responsive and was maintained following streptozotocin treatment, indicating that the K cells were spared the effects of streptozotocin.[151] These data suggest that it may be feasible to target the intestinal cells with vectors encoding the GIP-insulin transgene, or by *ex vivo* engineering intestinal cells in which glucose-sensitive promoters are driving insulin expression. However, an effective means of gene delivery to these cells needs to be developed for *in vivo* gene therapy, as these cells are present in the crypts of the gut, significantly impeding access to viral transduction.

Stem/progenitor cells

The considerable genetic manipulations that are required to convert non-β cells into efficient glucose-sensing, insulin-secreting cells have led other investigators into considering means of expanding adult or neonatal β cells or of harnessing the developmental potential of islet precursor cells and embryonal stem cells. However, despite the culture conditions and manipulations, commitment to β cells and insulin production has not always been consistent.[152–157] Much excitement has also surrounded observations that adult stem cells from bone marrow or from other tissues could 'transdifferentiate' into a number of other lineage-different cell types. Such stem cells have been described and sometimes physically isolated in the nervous system, pancreas, epidermis, mesenchyme, liver, bone, muscle and endothelium. Hematopoietic stem cells, in some studies, were proven able to yield endothelial, brain, muscle, liver and mesenchymal cells. In some studies, hematopoietic cells could also be generated from neuronal or muscle stem cells (reviewed in Wagers *et al*[88]). A number of issues, however, have tempered the enthusiasm with which these observations were initially greeted. The contamination of hematopoietic stem cells with mesenchymal precursors or the programming by growth factors in culture, and more recently, the phenomenon of fusion of stem cells with tissue cells are perhaps the most important variables to better test.[88,158] Recent developments, however, strengthen the belief that mesenchymal cells in bone marrow may be a multipotent source of cells.[85–87] This characteristic can be exploited; however, there are no data on whether such cells can be differentiated along the islet and β-cell lineage. Clearly, the ability to manipulate blood-borne progenitors into the β-cell lineage should provide a significant breakthrough for surrogate β-cell technology as insulin replacement.

Despite the current controversy and the serious ethical issues raised by cloning technology, it is likely that therapeutic cloning, under strict and defined conditions, will find its place in stem cell therapies.[159–161] In this regard, one possible means of propagating β cells or progenitors while avoiding the complications involved with the immune response could entail the removal of DNA or nucleus from somatic cells of a patient, its transfer into an enucleated embryonal stem cell, and its expansion into an appropriate β-cell lineage. While this remains highly speculative at present, the rapid pace of basic work in this area, despite restrictions, will likely yield insight into such manipulations.

Immortalization of islet cells with a β-cell phenotype has been attempted and successfully achieved. Insulin production, however, seems to be linked to terminal differentiation of the cell, an event normally reached with growth arrest. This problem has so far limited the utility of cell immortalization. Also, this approach carries with it the possibility of oncogenic transformation.[162–165]

Although still controversial, there are data indicating that mature human β cells can be induced to replicate under the effects of hepatocyte growth factor (HGF).[166–168] The limitation of this approach, however, rests on the loss of differentiation of the induced β-cell along with a substantial decrease in insulin production.[169] Conditional replication of non-human β cells has been achieved by placing the SV-40 T antigen under the control of an inducible promoter.[163] In these studies, β cells were able to replicate and to maintain differentiated function under inducible conditions. No data exist on whether such an approach is feasible in human β cells.

Propagation of islet precursor cells with subsequent genetic manipulation to commit them to the β-cell lineage and ultimately to β cells has also been considered.[156,170] To become feasible, this approach, however, requires a more complete understanding of the hierarchy of master regulatory transcriptional genes. Depending upon the cell type, PDX-1 overexpression can impart onto it a β-cell or a β-cell-like phenotype.[171–173] Indeed, Ferber *et al*[174] demonstrated that adenoviral gene transfer of a PDX-1 gene into liver resulted in insulin-expressing cells, although it was not clear if these cells were glucose-sensitive and were actually secreting the insulin in a timely fashion. Other important transcriptional regulators associated with differentiation of ductal epithelial cells into endocrine islet cells include the HNF family of transcription factors, PAX-4 and PAX-6, NeuroD/B2, Nkx 2.2 and Nkx 6.1.[173,175,176] Along with intracellular determinants, precursor cells require signalling from their environment to differentiate appropriately. A variety of polypeptide growth factors including insulin-like growth factors I and II,[177–181], prolactin,[182] placental lactogen,[183,184] parathyroid hormone-related peptide,[185,186] and, to a limited extent, TGF-α,[179,187,188] can promote pancreatic cell growth and islet cell proliferation. Hart and co-workers[189,190] colleagues have produced evidence suggesting that fibroblast growth factor (FGF) signalling is important for β-cell generation.[189,190] Strategies aimed at engineering β-cell progenitors from pancreatic ductular epithelium with FGF in the presence of a permissive PDX-1 expression could promote expansion of β-cell progentors or a differentiation of progenitors into a pre-β-cell lineage.

Another class of factors whose expression and production is associated with pancreatic regeneration has been identified.[191–193] The Reg-secreted protein, in particular, promotes increases in β-cell mass in rats that had undergone pancreatectomy.[194–196] The expression and secretion of another molecule that belongs to the Reg family of proteins, termed islet neogenesis-associated protein (INGAP), is upregulated in hamster islets where neogenesis was artificially induced.[197,198] The precise role of INGAP on β-cell proliferation and function, however, remains unclear.

Very recently, Bonner-Weir *et al*[199] have shown that it may be feasible to derive β-cell cluster buds from exocrine pancreatic tissue from which originate the ductular epithelial cells destined to become endocrine pancreatic islet cells. This approach is exciting in that mature, nonendocrine tissue of the pancreas need not be wasted during the process of islet isolation, but can be used in defined culture systems to generate islet progenitor cells for further manipulation, genetic or hormonal.

Thus, taken together, the transfer of combinations of genes encoding soluble and intracellular differentiation factors to stem/progenitor cells could become feasible once their precise role in the pathway of commitment and differentiation to β cells becomes clearer. However, β cells have a limited lifespan *in vitro*. To what extent apoptosis or senescence play a role in this is uncertain.

Nonetheless, a better understanding of cell cycle control in β cells or neonatal islet cells could lead to the discovery of molecules that could be exploited, in a conditional manner, to promote growth *in vivo* and maintenance or extension of lifespan, both *in vitro* and *in vivo*. Possible means include the transfer of cyclin-dependent kinases, proreplication and mitotic factors and/or telomerase to promote expanded cell lifespan, all under regulatable promoters. Such an approach could achieve the expansion of semicommitted or fully committed islet precursor cells, or early β cells. Combined with xenogeneic donor manipulation, these interventions could provide an almost limitless supply of β cells for transplantation. The recent success in knocking in a non functional α(1,3)-galactosyltransferase in order to generate a transgenic pig deficient for this enzyme may forecast the inclusion of modalities in which transgenic porcine islets can be used instead of allogeneic human islets for transplantation.[200,201] The importance of this breakthrough is underscored by the fact that the major target of xenoreactive antibodies, which promote an acute rejection of porcine tissues, is the epitope that is synthesized by this enzyme. While this is the major porcine xenoantigen, it is almost certain that other minor porcine epitopes will contribute, perhaps not to acute rejection, but to delayed or chronic xenograft rejection and these are challenges that must be surmounted in the future.

Looking towards the future

Clearly, many unexplored avenues await to be negotiated with the technology in existence today. First, combinations of immunosuppressive transgene cassettes should be used to transduce intact islets in culture in parallel with efforts at identifying the vector with the least immunogenicity. Second, it will be impossible to infect every single β-cell within intact islets; consequently, it may be more effective to target soluble molecules that activate β-cell death. In this way, vectors that may not be islet transduction-efficient, yet non-immunogenic and stably integrating, can still be used effectively when expressing multicistronic transcripts encoding inhibitors of multiple proapoptotic pathways, for example. Cell therapy will very likely include stem and progenitor cells as replacement or surrogate β cells. The biology of these cells is now beginning to be unraveled, and in parallel with advances in tolerance induction and gene transfer technology may yield an efficient means of promoting not only islet allograft or xenograft survival, but may even lead to operational tolerance in an autoimmune background.

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Gene therapy technology applied to disorders of glucose metabolism: promise, achievements, and prospects

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Gene transfer technology has spawned an entire realm of clinical investigation, collectively referred to as "gene therapy." The feasibility and achievements of gene therapy to prevent and treat glucose homeostasis disorders, with particular emphasis on diabetes mellitus, are evaluated in this review. While a considerable amount of effort has yielded gene delivery vectors based on adenoviral, retroviral, and herpes simplex virus DNA, the number of successful clinical applications has not been as impressive. Despite the number of successes in vitro and in animal models, preliminary safety trials in humans have not yet been attempted. The current state of this science, outlined here, underlines the necessity of marrying gene transfer technology with cell therapy. The ex vivo transfer of gene combinations into a variety of cell types will likely prove more therapeutically feasible than direct in vivo vector transfer. Current efforts aimed at assessing the future of gene therapy for diabetes must, at the very least, take into account the importance of moving successful methods into human safety trials.

INTRODUCTION

Diabetes mellitus refers to a multitype disorder of glucose homeostasis defined by the loss of insulin production and/or the insensitivity of peripheral tissues to the effects of insulin. Generally, the disorder falls into two categories: type 1 and type 2 (1–4). The loss of insulin due to the autoimmune destruction of the β cells defines type 1 diabetes mellitus. Impaired glucose sensitivity and/or insulin production by pancreatic β cells in the presence or absence of poor sensitivity to the effects of insulin in the periphery define type 2 diabetes, which is commonly diagnosed in adults. Often, obesity is a significant risk factor for type 2 diabetes (5–7). Today, the conventional treatment for type 1 diabetes remains insulin replacement. Pharmacologic agents acting at the level of the β cell to improve glucose sensitivity and insulin production, along with drugs that can sensitize the peripheral tissues to the effects of insulin, are the forefront of the effort aimed at restoring good glucose control in type 2 diabetics. In addition, several bioactive peptides have also been discovered that have potent effects on insulin production and are currently in various stages of clinical development (8–10). However, insulin replacement and the various pharmacologic agents cannot be considered "cures." Despite the significant improvements in glycemic control, these strategies are unable to achieve permanent, normal, physiological glucose control. The consequences include diabetes complication-associated morbidity and mortality (11). In fact, nonphysiologic glucose control is a predominant risk factor for cardiovascular, renal, and ophthalmic diseases. Where traditional pharmacological therapies fall short, bioengineering strategies offer an alternative.

Gene transfer technology is the cornerstone of gene therapy, widely cited as an alternative approach to, or as a complement of, traditional pharmacotherapy in several human disorders. Indeed, a number of studies in animals have demonstrated the potential of this technology in type 1 diabetes (12,13). While advances in metabolic engineering of cells have not kept pace with those in the field of immune regulation; nonetheless, several approaches for engineering surrogate β cells are equally promising. In this review, we outline the technologies that have been used in gene therapy strategies for disorders of glucose homeostasis, with a special emphasis on diabetes mellitus. We illustrate the successful applications of gene transfer technology and current limitations. We conclude with some proposals that could serve as the basis of further investigation.

DISORDERS OF GLUCOSE HOMEOSTASIS

While several human disorders have impairments in glucose homeostasis (hyperglycemia/hypoglycemia) as their symptoms; often, this is a consequence

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of the disorder and transient. Primary chronic hyperglycemia is synonymous with type 1 and type 2 diabetes mellitus, and type 2 diabetes is closely associated with obesity. To uncover targets of intervention, it is important to understand the molecular mechanisms of the etiology and progression of these disorders.

Type 1 diabetes is an autoimmune disorder whose onset occurs at a very early age and, in most patients, results in pancreatic β cell impairment or destruction by the time of diagnosis, which requires insulin replacement. It is a T cell-driven disease that relies on the presence of specific HLA alleles that are often found clustered in families (1,2,14,15). Despite the confirmation that the disorder has a genetic basis, the significant concordance between twins and the mapping of susceptibility loci, type 1 diabetes is thought to involve an environmental trigger (2,15). The T cell specificity for pancreatic β cell antigens exposes an underlying defect of central tolerance mechanisms and, very likely, dysfunctional peripheral immunoregulation.

In contrast, type 2 diabetes is consequence of several, often overlapping, physiological and cellular defects, with only sporadic evidence of an autoimmune component (16). Type 2 diabetes mellitus is associated with insulin deficiency and insulin resistance. It is still unclear where the primary defect lies and which of the two is a secondary response, although many studies support insulin deficiency as secondary to peripheral resistance (3,17). Genetic and environmental factors contribute to the etiology of type 2 diabetes mellitus, with the concordance rate in identical twins at 90% with variable prevalence among different populations. More than 50 genes have been identified that are involved in glucose homeostasis, which suggests that the cause of type 2 diabetes is heterogeneous and will likely involve defects in many pathways that play a role in insulin expression, secretion, and sensitivity and that even this alone will define only a subset of cases (3,17).

Obesity is probably the most important environmental risk factor for type 2 diabetes. Obesity induces insulin resistance in both humans and animal models of type 2 diabetes (5,18,19). Several genetic defects have been discovered that implicate the leptin pathway as a key regulator of satiety and obesity (18,19). Leptin-deficient and leptin receptor-deficient mice and rats are obese and exhibit many characteristics of type 2 diabetes to varying degrees. The importance of leptin in human obesity, insulin resistance, and type 2 diabetes, however, remains to be determined, although there is no current evidence to support leptin defects as causal of human obesity or diabetes (18,19).

A role for free fatty acids in insulin resistance and type 2 diabetes etiopathogenesis has been proposed by a number of studies, following the observation that chronic elevation of serum-free fatty acid concentrations in obese or diabetic individuals is associated with decreased glucose uptake into peripheral tissues (20–22).

GENE THERAPY TECHNOLOGY

It is evident from the physiology that some tissues and cell types are targetable by, and exploitable for, gene engineering to achieve one or more desired therapeutic benefits. For type 1 diabetes, the cells of the immune system can be targets of molecular vehicles that carry genes or proteins able to induce immunosuppression in a spatially confined manner and with restricted tissue distribution. In parallel, transplantation of insulin-producing tissues can be engineered to resist rejection with no need for the chronic administration of toxic immunosuppressive drugs. Additionally, understanding the process of T cell maturation can assist in designing gene engineering strategies that can prevent the onset of autoimmunity.

While it may not be readily apparent why gene therapy can or should be applied to type 2 diabetes, most of the pharmacologic interventions used to treat insulin resistance (a preface to overt diabetes) eventually fail, which suggests that a targeted approach may be more able to prolong the time to onset of insulin resistance or prevent it altogether. While "insulin resistance" genes have yet to be identified, type 2 diabetes has been associated in rodents and humans with specific mutations/polymorphisms of glucoregulatory, glucose-sensing genes, or is preceded by obesity and insulin resistance that may or may not be underlined by genetic susceptibility (3,23), which suggests that gene therapeutic strategies aimed at type 2 diabetes should be based on a knowledge of the biochemistry of glucose-sensing, insulin sensitivity, and fuel metabolism. Targeting one or more of these pathways may achieve the identical results as current pharmacologic therapy, with no risk of the toxicity inherent in some of the more aggressive and potent agents currently in clinical use.

To deliver genes into cells and tissues, it is necessary to identify the "engineerability" of a cell; whether it is a dividing cell, differentiating cell, cell with limited lifespan, or cell with high metabolic activity. These factors will determine the choice of vector system that will facilitate the conferring of an exogenously demanded activity to a cell. Currently, vector systems fall into two broad categories, each with their advantages and limitations. We will describe these in the context of gene therapy for diabetes mellitus. Table 1 illustrates the general properties of the two vector categories, viral and nonviral.

Viral Vectors

The generation of viral gene delivery vectors requires two major components: vectors encoding a complete, or components of, viral genome sequence that is engineered in such a manner that no replication-competent particles can be generated. Additionally, the gene or genes that achieve the desired therapeutic or prophylactic objective are supplied as part of the viral sequence or separately in another DNA sequence. The second component is a cell line that can efficiently produce recombinant virions that do not have the capacity to replicate once they infect a host. Several strategies are currently employed to generate replication-defective adenoviral vectors (Figure 1) (24–34), and some are commercially available. Table 1 outlines the reasons adenoviruses are versatile. Their most important feature is the ability to generate these vectors in high titers and to infect a broad range of cell types (24,25). It is therefore not

surprising that the greatest number of gene therapy approaches in vitro and in vivo have been carried out with adenoviruses. The major limitation of these vectors is their immunogenicity, which is very likely dependent on the presence of expressed sequences in the vector backbone (24,25). If the aim is to generate a state of localized immunosuppression, then such a feature is intuitively counterproductive. Several strategies have succeeded in generating "gutless" adenoviral vectors, but to date, it has been challenging to grow these vectors to high titer without the presence of contaminating helper virus (24,25). Adeno-associated vectors offer advantages over adenoviruses, and recent data suggest their potential benefits (35–40). What is clear is that the utility and versatility of novel viral vectors are ultimately compared to that of adenoviruses; generally, adenoviruses are the benchmark.

Retroviral vectors were among the earliest choice vectors in gene therapy models. They easily infect replicating cells and integrate into the host genome. Actively replicating immune cells, especially T cells, were among the first cell types to be transduced by retroviruses. Their generation is straightforward, and several cell lines and processes are currently commercially available. Primarily based upon the Moloney murine leukemia virus (MMLV) genome, retroviral vectors have evolved into embodiments of avian, bovine, feline, equine, and human retroviruses (27–30,32,34).

The properties of human immunodeficiency virus (HIV)-1 were exploited to develop the first vectors of the *Lentiviridae* family. Lentiviral vectors

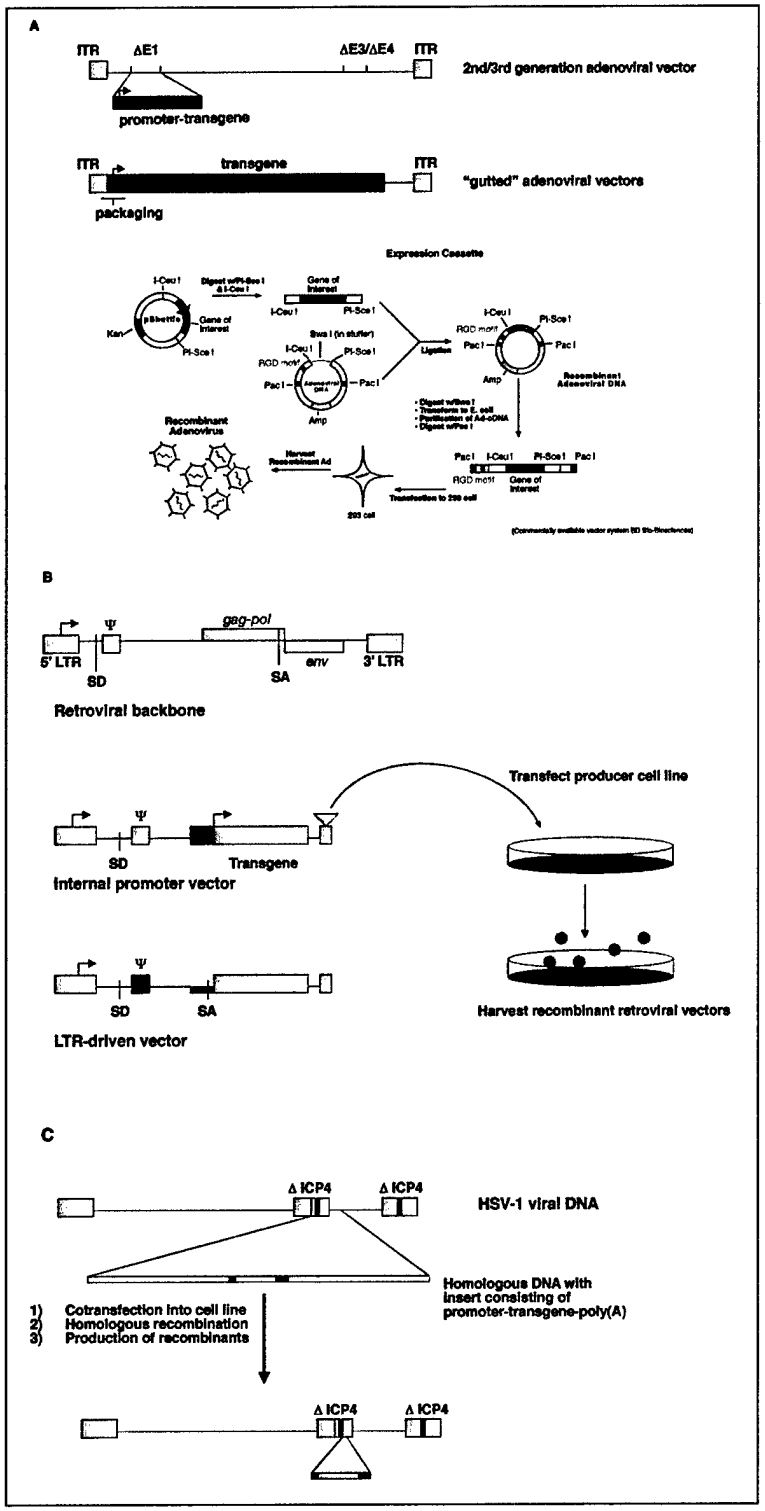


Figure 1. Construction of viral vectors. (A) Adenoviral vectors. Cell lines expressing adenoviral helper and packaging functions are transfected with transfer plasmids encoding the transgene of interest. Recombination of homologouos sequences occurs within the cells. Newer developments take advantage of recombination within appropriate bacterial hosts, minimizing nonrecombinant vector production in cells. (B) Retroviral vectors. Transfer plasmids encoding transgenes are transiently transfected into packaging cell lines. (C) Herpes viral vectors. To construct a vector that is ICP4- and ICP22- (deletions in crucial genes that are necessary for replication competency), ICP4-viral DNA is transfected into a producer cell line with a transfer plasmid in which the transgene cassette is cloned inside the deleted ICP22 locus.

offer low to no immunogenecity, but their cell range is limited, and the titers achievable on a packaging cell basis are significantly lower compared to adenoviruses. In the diabetes context, they can infect cells that are poorly transducible by adenoviral vectors and offer the advantage of stable genomic integration. This is a desired feature in approaches aimed at engineering cells that do not divide, differentiate, and are long-lived (27–30,32,34).

A limitation of all the aforementioned vectors is the transgene size that can be accommodated inside the vector backbone. Where strategies envisage multicistronic transgenes and/or promoters of complex (and long length) structure, solutions are provided by the herpes simplex viruses (26,28,41). While earlier generations of these vectors were toxic to transduced cells, current embodiments do not promote significant levels of cell toxicity in vitro or in vivo (42–46). A disadvantage is the cumbersome process required to generate significant titers, but ongoing efforts at large-scale vector production promise to overcome this limitation (42–46).

Nonviral Vectors

While viral vectors are versatile for almost all gene therapy applications, the immunogenicity, toxicity, potential for replication competency, the potential of wild-type helper virus carryover during production, and the sheer logistics required to maintain good manufacturing procedures/good laboratory procedures facilities for clinical-grade vectors are considered to offer more challenges than solutions. Nonviral vectors offer an inexpensive alternative. Traditionally, the transfer of naked DNA plasmids was synonymous with nonviral vectors; however, several recent developments have welcomed new types of gene transfer vehicles that contain no viral elements.

Naked recombinant DNA plasmid generation is rapidly scalable with considerably lower cost compared to viral vectors. Newer generation plasmid vectors have been designed to either stimulate immune responses or to have a low immunogenic profile (33,47–51). Additionally, multicistronic vectors are easily achievable compared to challenges to generate the same in viral vectors. Nonetheless, naked DNA cannot attain stable transgene persistence in transduced cells, and high concentrations are often required in multiple dosings (Table 1).

Newer approaches have succeeded in exploiting amino acid sequences that act as a cell entry facilitator of intact proteins. These peptide transduction domains (PTDs) are derived from viral nuclear import proteins and, unlike viral and plasmid DNA vectors that require gene transcription and translation for transgene expression, the PTDs are fused to the protein of interest. PTD-fused proteins can be readily generated in high yield with relatively minimal logistical requirements (52–56). The nature of the PTD domain can be modified to improve cell-type targeting. Their limitation, however, lies in their relatively short half life, as they are subject to proteasome-dependent proteolyis. Whatever the choice of vector, all have been tested in the context of gene therapy for diabetes, with varying outcomes.

THE PROMISE, ACHIEVEMENTS, AND PROSPECTS

The most obvious form of physiological glucose regulation in diabetic individuals has been insulin replacement. It is now evident that pharmacologic insulin replacement, although able to achieve tight glucoregulation, cannot prevent the complications. The alternative that many have aspired to has been to facilitate islet transplantation or to engineer surrogate β cells. While the recent successes achieved by glucocorticoid-free immunosuppression have propelled islet transplantation closer to routine clinical consideration, pharmacologic immunosuppression is in fact detrimental to the long-term survival of islet transplants, necessitating a local form of immunosuppression that is adequate to control the rejection of the transplant without affecting the host immune response at a systemic level.

For diabetes, the choice of approach depends on the very nature of the underlying defect. In type 1, the objective is to educate the host immune system to eliminate autoreactive T cells before the clinical onset of the disease or to re-

Table 1. General Characteristics of Gene Delivery Vehicles

Vector type	Advantages	Disadvantages
VIRAL		
Adenovirus	Choice vector for pilot proof-of-principle experiments; high titers easily obtained; almost all cells and tissues are transducible; and cell retargeting is possible.	Immunogenic in vivo; nonstable transduction.
Adeno-associated virus (AAV)	Site-specific, stable integration achievable; almost absent immunogenicity; and many cell types transducible.	Time for transgene expression can be days.
Moloney murine leukemia virus (MMLV)-based retrovirus	Stably integrating vector in rapidly dividing cells; cell-type retargeting possible; and good titers are obtainable.	Subject to chromosomal position-effect sensitivity of, as well as methylation and cytokine effects on, gene expression.
Lentivirus	Nonimmunogenic, stably integrating; choice vector for nondividing, noncycling cells; good titers obtainable; data support absence of replication-competent-recombinant vector particles in stocks.	Clinical safety concerns with human immunodeficiency virus (HIV)-1-based vectors.
Herpes simplex type-1 virus	Large genome available for multiple large size cistrons; good persistence in many cell types; and cell-type retargeting possible.	Inherent toxicity.
NONVIRAL		
Plasmid DNA	Easy to engineer, grow, and purify; and multicistronic variants are easy to engineer.	Poor persistence, nonspecific cell targeting, and poor dissue diffusion.
Cationic liposome	Easy to manipulate to deliver plasmid DNA to almost all cells and tissue. Nonimmunogenic; cell-type nonspecific, and cell-type retargeting possible.	Poor control of diffusion kinetics.
Peptide fusion domains	Many cell-types transducible; high-level protein/peptide import; intact proteins/ peptides delivered; not subject to gene regulation; targeting of specific proteins possible; high-level peptide production easily achievable; and no reported immunogenicity.	Short half life; subject to proteolytic degradation; and large amounts require some time to generate.

place the lost β cells with either intact islets or surrogate β cells. In type 2 diabetes, the objective is to initially restore metabolic control by regulating fuel metabolism, insulin sensitivity and production, and glucose uptake.

In general, there are two strategies that can be employed to generate a cell or gene drug: ex vivo engineering or in vivo delivery. In the ex vivo approach, a cell that will be used as a transgene factory is isolated from the host and manipulated in the laboratory. This may or may not involve cell expansion. Most gene therapy strategies employ this approach. The in vivo interventions are more difficult because the targeting of the gene delivery vehicle becomes an issue and is currently difficult to achieve, unless the targeted site is where the correction is being made and is easily accessible.

Many of the approaches for type 1 diabetes involve gene transfer to cells ex vivo. Consequently, one must take into the account the nature of the cell to employ an appropriate delivery vector. The primary target cell type has been the β cell in the form of intact islets for subsequent transplantation and immune cells of different types, most commonly the antigen-presenting cells or T lymphocytes. The in vivo approaches could involve targeting to the pancreas to organs and sites involved in immune stimulation, such as the thymus and the peripheral lymphoid organs.

To introduce a therapeutic transgene to β cells ex vivo, the delivery vehicle must be able to transduce these almost nondividing cells and to remain in the cell for its entire lifetime. The most

Table 2. Potential Intervention Strategies

Type 1 Diabetes Mellitus

Gene Therapy

Facilitation of islet transplantation

- Ex vivo transduction with vectors encoding immunoregulatory transgenes (co-stimulation blockade, cytokine antagonists, and apoptosis inhibitors).
- Infusion of antiapoptotic/immunoregulatory peptides into transplant recipients pre-transplantation and post-transplantation.
- In vivo administration to transplantation site of vectors encoding trophic factors for islets.

Tolerance Induction

- In vivo immunomodulation using vectors encoding immunosuppressive/ tolerogenic transgenes.
- In vivo adminstration of vectors encoding putative autoantigens.

Cell Therapy

Surrogate β cells

- Embryonic stem cells differentiated into cells with β cell characteristics.
- In vivo administration of vectors encoding genes promoting β cell phenotype to pancreatic/hepatic cells.
- Expansion of progenitor cells ex vivo.
- Therapeutic cloning technology.

Type 2 Diabetes Mellitus

Gene Therapy

Prevention of insulin resistance

- Transduction of adipose and/or muscle with vectors encoding insulin-sensitizing genes and proteins/peptides.
- Transduction of the liver with vectors encoding gluconeogenic-regulatory genes.
- Transduction of peripheral, insulin-sensitive tissues with vectors encoding antagonists to cytokines inducing insulin-insensitivity.

Anti-obesity strategies

- Targeting the hypothalamic-adipose axis (i.e., leptin, neuropeptide Y, and satiety factors).
- Transduction of adipose with vectors encoding anti-lipolytic genes.

Cell Therapy

Surrogate β cells

- Embryonic stem cells differentiated into cells with β cell characteristics.
- In vivo administration of vectors encoding genes promoting β cell phenotype to pancreatic/hepatic cells.
- Expansion of progenitor cells ex vivo.
- Therapeutic cloning technology.

versatile delivery vehicles are replication-deficient viral vectors, and Table 1 lists their advantages and disadvantages. In subsequent sections, their utility in various experimental models of type 1 diabetes gene therapy will be discussed. For peripherally derived antigen-presenting cells, the requirement for long-term expression may not be as stringent as for the β cells, primarily because the source of these cells (i.e., peripheral blood) is easily accessible. In this instance, novel approaches in addition to viral gene delivery can be employed. An example is the newly discovered peptide delivery vectors

Table 3. Current Approaches for Type 1 Diabetes Gene and Cell Therapy

Gene Vectors that Transduce Islets (with References)	Genes that Promote Islet Allograft/ Xenograft Survival In Vitro and In Vivo and/or β Cell Survival In Culture	Other Gene/Cell Therapy Approaches to Prevent/Abrogate Autoimmunity and/or Promote Islet Allograft/Xenograft Survival
Plasmid DNA (173,193–195)	**Anti-apoptotic genes**	**Bone marrow transplantation/ chimerism induction** (178,243–253)
Adenovirus (196–209)	bcl-2 (214,216,224–226)	
AAV (210–219)	bcl-x_L (52,227)	
MMLV-based retrovirus (220)	heme oxygenase-1 (228–230)	**Antigen-presenting cell (APC) transfer**
Lentivirus (221–223)	dominant negative protein kinase C delta (231)	class I MHC (163)
Herpes simplex virus (224,225)	dominant negative MyD88 (232)	autologous dendritic cell (DC) transfer (254)
Cationic liposomes (194,195,204)	IGF-I (233)	
Peptide fusion domains (52,53,56)	Iκβα super-repressor (209)	**Co-stimulation blockade**
	Hsp70 (234)	soluble ICAM-1-Ig (255)
	A20 (205)	CTLA-4Ig (256–260)
	PEA-15 (52)	OX40Ig (259)
	catalase (235,236)	
	manganese superoxide dismutase (237,238)	**Cytokines**
		IL-10 (239,261-263)
	Cytokines	IL-4 (262,264)
	IL-4 (221) (although one report demonstrated no protection) (196)	soluble IFNγ receptor (187,265)
		TGFβ (266)
	IL-1 receptor antagonist protein (208)	
	IL-12p40 (197)	**Autoantigen transfer**
	viral IL-10 (198,239)	glutamic acid decarboxylase (GAD) (267)
	IL-10 (240) (one report did not show protection) (196)	
	TGFβ (240) (one report showed negative results) (241)	**Others**
		adenovirus E3 proteins (268)F
		orally administered putative autoantigens (insulin and GAD) (269–271)
	Immunoregulatory genes	CD152 (272)
	Indoleamine 2,3-dioxygenase (215)	
	CTLA-4Ig (173)	
	Fas ligand (199) (although in several reports, the Fas ligand was not protective) (242)	
	adenoviral E3 genes (200)	

that can introduce proteins in their native forms across the cell and the nuclear membrane (53,57,58).These peptide-protein conjugates can be generated quite readily and have been shown to transduce many cell types in culture, including islet β cells (56).

However, in vivo gene delivery is more challenging and will require appropriate targeting. The direct injection of adenoviral vectors into the pancreas of rats has been attempted, as has plasmid DNA and adenoviral gene delivery to the thymus (59). Whether these approaches can become more efficient or clinically useful remains to be explored.

Type 2 diabetes, on the other hand, may be better suited for in vivo gene delivery at either the sites of metabolic defects or at sites where the biochemistry can be manipulated to restore normal glucose levels. For example, because insulin action on glucose metabolism is mainly on skeletal muscle and adipocytes, one could target large muscle groups or sites of fat deposit with viral vectors (Table 1) to improve insulin sensitivity or to substitute skeletal muscle as a site of increased glucose uptake where other sites are defective. This approach can entail the use of direct injection of viral vectors or, as recently identified (60,61), injection of skeletal muscle stem cells that, once obtained from an autologous host, could be modified ex vivo with viral

gene vectors, expanded, and transplanted back into a large muscle group. However, pancreatic defects will probably require either direct injections into the pancreatic vasculature or islet/surrogate β cell transplantation. Whatever the defect, type 2 diabetes gene therapeutics will most likely be patient-specific because the defects among the patients are not expected to be identical.

Table 2 lists the general strategies aimed at therapeutic or prophylactic end points. Several vectors and transgenes, used to confer immune privilege and to bolster intrinsic defenses of the β cell, are listed in Table 3. Note that all of the studies we will describe have been performed in human or rodent islet/β cell cultures or rodents in vivo. The results have been mixed. To date, there is no single transgene that has conferred significant long-term protection to transplanted islets in allogeneic diabetic rodent recipients. Moreover, although monogenic gene transfer to human or rodent islets has thus far been unable to prevent autoimmune rejection, as evidenced in rodent models of autoimmune diabetes, it is possible that multigenic gene transfer, which combines the properties of two or more transgenes in protecting islets at multiple levels or uses two or more vectors each encoding different genes, may succeed. Additionally, not all "immunoregulatory" genes have been tested. In the absence of these data, it would be unwise to view the transplantation of gene-engineered islets expressing multiple immunoregulatory genes as unfeasible or without potential. These studies must be carried out before reaching any general conclusion.

In parallel with the transplantation of gene-engineered islets, several attempts have been made to generate surrogate β cells from rodent or human progenitors. The most popular approach has been to convert hepatocytes or hepatocyte cell lines into glucose-sensitive insulin-producing cells for the purpose of transplantation. Direct in vivo administration of such vectors has also been attempted. Adenovirus has been the choice vector in these strategies, and several transgenes have been tested in the context of putative glucose-sensitive promoter elements.

Hepatocytes are particularly attractive because they can easily engraft in the liver, and because they possess glucose-sensing molecules identical to the pancreas [e.g., GLUT2 and glucokinase (GK)]. Furthermore, one can exploit several hepatocyte gene promoters that are sensitive to glucose to engineer insulin transgenes to be glucose concentration-sensitive. The disadvantages of hepatocytes, however, lie in their inability to secrete the insulin in a glucose-responsive fashion and their lack of PC2 and PC3 pro-convertases and carboxypeptidase H, required to process proinsulin to its mature form (62,63). Despite these limitations, Groskreutz et al. (64) were able to engineer human proinsulin to contain furin cleavage sites at the natural sites of proinsulin processing. Furin is a ubiquitous protease that is also found in hepatocytes. This form of proinsulin was properly processed into active and mature insulin in transfected human kidney cells and rat primary myoblasts and possessed insulin receptor binding and activation activity similar to native insulin. Recent efforts have confirmed that hepatocytes are equally efficient at producing and processing this furin site-modified proinsulin into a mature, bioactive form (65). The need to express proinsulin in a glucose-dependent manner compelled Mitanchez et al. (66) to engineer transgenic mice expressing the human proinsulin cDNA or the furin cleavage site variant under the transcriptional control of the glucose-responsive liver isoform pyruvate kinase (LPK) promoter-enhancer elements. Insulin was expressed in the liver, kidney, and gut of these transgenic mice, and immunoreactive C-peptide was readily detectable in serum. Moreover, its levels were sensitive to levels of carbohydrates (66). More studies on this system using adenoviral gene transfer to the liver have recently been reported by Thule et al. (67,68), who have shown its potential in a streptozoticin-treated rat model. Very recently, Lee and colleagues (69) were able to achieve the complete restoration of normoglycemia in diabetic rats following recombinant adeno-associated virus (rAAV) gene transfer of a single-chain insulin transgene under the control of the LPK pro-

moter. Additionally, they obtained the same success in non-obese diabetic (NOD) mice. While these data are very compelling, the kinetics of changes in serum glucose concentration are not in harmony with the known kinetics of transcriptional activation of the LPK promoter. Furthermore, insulin secretion by hepatocytes is not glucose-regulated. In a related approach, Chen et al. (70,71) demonstrated in hepatoma cell culture that adenoviral gene transfer of insulin driven by the glucose-6-phosphatase promoter resulted in glucose-responsive, self-limiting insulin production, although these data are not clear on whether this approach can be successful in vivo.

Despite these promising approaches, much more work is needed to make hepatocytes into fully surrogate β cells. First, their response to glucose is not as rapid as that found in β cells. Second, the liver-specific glucose-sensitive promoters have elements that respond to hormonal and metabolic signals, which can impede, attenuate, or abrogate the desired objective of tight glucose regulation. For example, instances of hyperglucagonemia, which are to be expected in the absence of functional endogenous β cells in diabetics, will most likely attenuate or repress the LPK promoter and other promoters such as GK (72–74). Third, glucose-dependent transactivation of the LPK promoter requires the GK-dependent phosphorylation of glucose, an activity that is insulin-dependent (66). Other promoters have been suggested, such as phosphoenolcarboxykinase (PEPCK), but this promoter is activated by glucagon and inhibited by insulin, which may not result in the desired kinetics of physiological glucoregulation (75,76). It is possible that a combination of promoter elements from different glucose-responsive hepatic genes may be required to create an optimal synthetic promoter to drive hepatic insulin expression in a true glucose-sensitive fashion.

In addition to hepatocytes, one of the earliest approaches involved the genetic modification of an adrenocorticotrophic hormone (ACTH)-secreting neuroendocrine cell line (AtT20) to secrete human proinsulin where proinsulin expression was under the control of a viral promoter (77). The advantage of these cells was they shared some features with β cells; namely, their possession of secretory granules and the expression of the PC2 and PC3 proconvertases and carboxypeptidase H, which cleave proinsulin to its mature form (78). Further manipulations of the cell line resulted in derivatives that expressed GK and GLUT2, the high Km glucose transporter of β cells. These cells were able to secrete insulin in a glucose concentration-dependent manner and responded to insulin secretagogues potentiated by glucose, which are features that are inherent in β cells (79). Despite this, the basal insulin release by these cells is higher than that of β cells. Another concern in the use of these cell lines is the effect of GLUT2, which is permissive for the secretion of ACTH and possibly other pro-opiomelanocortin-derived hormones in a glucose-dependent manner (66), potentially leading to iatrogenic Cushing's syndrome. Furthermore, the cytomegalovirus promoter may become inactive in vivo (80).

More recently, tissue-specific promoters have been exploited to engineer cells to express insulin in cells that are not targets of autoimmune destruction. Lipes et al. (81,82) have expressed insulin in the anterior pituitary of NOD mice under the control of the pro-opiomelanocortin promoter. Insulin was expressed, stored in secretory granules, and exhibited regulated secretion. Moreover, the transplantation of transgenic anterior pituitary tissue to NOD mice was able to partially restore normoglycemia with no signs of immune rejection (81,82). However, it was unclear if insulin secretion was glucose concentration-dependent in these cells. Very recently, an ingenious approach harnessing intestinal K-cells as surrogate glucose-responsive insulin producers was demonstrated. In this approach, transgenic mice expressing human insulin under the control of the gastrointestinal inhibitory peptide (GIP) promoter were generated. These mice expressed and secreted insulin from intestinal K cells in which the GIP promoter is active. Insulin secretion in these mice was glucose-responsive and maintained following streptozotocin treatment, indicating that the K cells were spared the effects of streptozotocin (83). These data suggest that it may be feasible to target the intestinal cells with vectors encoding the GIP-insulin transgene or by ex vivo engineering of intestinal cells in which glucose-sensitive promoters are active driving insulin expression.

EXPANDING β CELLS OR β CELL SURROGATES

The considerable genetic manipulations that are required to convert non-β cells into efficient glucose-sensing, insulin-secreting cells have led other investigators to consider means of expanding adult or neonatal β cells or of harnessing the developmental potential of islet precursor cells to commit them to the β cell lineage. Even disregarding possible ethical concerns, the successful isolation of purified islet stem cells obtained from the fetus or neonate has remained elusive. Where precursor cells have been identified, commitment to β cells and insulin production has not always been consistent (84–88). Recent data indicate that embryonal stem cells can also assume insulin-producing properties (89). If a stable β cell-like phenotype can be maintained for these cells in culture and following transplantation, then they could be a source of renewable surrogates for β cells following manipulations to expand them.

Despite the current controversy and the serious ethical issues raised by cloning technology, it is likely that therapeutic cloning, under strict and defined conditions, will find its place in stem cell therapies (90–92). In this regard, a possible means of propagating β cells or progenitors, while avoiding the complications involved with the immune response, could entail nuclear transfer approaches. In this method, the nucleus (containing donor DNA) from somatic cells of a patient may be transferred into an enucleated embryonal stem cell that can be expanded into an appropriate β cell lineage. However, the issue of autoimmunity will remain. While this is highly speculative at present, the rapid pace of basic work in this area, despite restrictions, will likely yield insight into such manipulations.

The immortalization of islet cells with a β cell phenotype has been attempted and successfully achieved. Insulin production, however, seems to be linked to the terminal differentiation of the cell, an event that is normally reached with growth arrest. This problem has so far limited the utility of cell immortalization. This approach also carries with it the troublesome possibility of oncogenic transformation (93–96).

Although still controversial, there are data indicating that mature human β cells can be induced to replicate under the effects of hepatocyte growth factor (HGF) (97–99). However, the limitation of this approach rests on the loss of the differentiation of the induced β cell, along with a substantial decrease in insulin production (100). Conditional replication of nonhuman β cells has been achieved by placing the simian virus 40 (SV40) T antigen under the control of an inducible promoter (94). In these studies, β cells were able to replicate and to maintain differentiated function under inducible conditions. No data exist on whether such an approach is feasible in human β cells.

The propagation of islet precursor cells with subsequent genetic manipulation to commit them to the β cell lineage and ultimately to β cells has also been considered (88,101). For this approach to become feasible, however, a more complete understanding of the hierarchy of master regulatory transcriptional genes is required. Nevertheless, some progress has been made following the discovery that the absence of pancreatic duodenal homeobox (PDX)-1 (also known as IDX-1, IPF-1, STF-1, and IUF-1), a homeodomain transcription factor, results in pancreatic agenesis (102). In addition to differentiation of primitive pancreatic epithelium, PDX-1 appears to be important in glucose-sensitive transcriptional transactivation of the insulin gene and GLUT2, GK, and somatostatin (103–106). Depending on the cell type, PDX-1 overexpression can impart onto it a β cell or a β cell-like phenotype (102,107,108). Indeed, Ferber et al. (109) demonstrated that the adenoviral gene transfer of a PDX-1 gene into the liver resulted in insulin-expressing cells, although it was unclear whether these cells were glucose-sensitive (109).

The transfer of combinations of genes encoding soluble and intracellular differentiation factors could become feasible once their precise role in the pathway of commitment and differentiation becomes clearer. However, β cells have a limited lifespan in vitro. To what extent apoptosis or senescence play a role in this is uncertain. Nonetheless, a better understanding of cell cycle control in β cells or neonatal islet cells could lead to the discovery of molecules that could be exploited, in a conditional manner, to promote growth in vivo and maintenance or extension of lifespan, both in vitro and in vivo. Possible means include the transfer of cyclin-dependent kinases, pro-replication and mitotic factors, and/or telomerase to promote expanded cell lifespan, all under regulatable promoters. Such an approach could achieve the expansion of semi-committed or fully committed islet precursor cells or early β cells. Combined with xenogeneic donor manipulation, these interventions could provide an almost limitless supply of β cells for transplantation.

ISLET OR SURROGATE β CELL TRANSPLANTATION

Whatever the identity of the insulin-replacement cells (intact islets, ex vivo-generated β cells, or surrogate β cells), unless they are of autologous origin, they will need a defense against allogeneic, xenogeneic, and autoimmune attack. There are two levels at which these defenses can be deployed: (*i*) extracellularly, by the production and secretion of immunosuppressive and/or anti-apoptotic gene products, and (*ii*) intracellularly, where anti-apoptotic protein production could prevent intracellular pathways triggered by extracellular death ligands. Many approaches have been tested, and all are listed in Table 3.

APPROACHES FOR TYPE 2 DIABETES

Being the predominant risk factor for insulin resistance, obesity is an important target by which the eventual progression to type 2 diabetes can be prevented. Obesity is, itself, a multifactorial syndrome caused by genetic and envionmental factors. While the simple solutions to obesity are to consume in moderation, avoiding high-calorie foods on a long-term basis, and physical exercise for morbidly obese individuals or in those noncompliant to dietary regimens, gene drugs may be the alternative. The primary objective in these gene drugs would be to reduce satiety levels and to regulate the amount of fat. One of the principal regulators of body fat and a fuel sensor is leptin.

Leptin was initially identified as the gene whose mutated variants resulted in the obese phenotype of obese (ob/ob) mice. Leptin encodes a secreted protein with weight-reducing, satiety-suppressive, and insulin- and glucose-lowering effects (110–114). In a small group of morbidly obese humans, the leptin gene was also found to be mutated, but evidence suggests that almost all forms of obesity in humans are not due to leptin defects (115–118). A series of studies demonstrated that viral gene transfer of leptin to rodent models of obesity was effective in accelerating weight reduction, decreased adipocyte and fat deposits, enhanced fatty acid oxidation, and lowered expression of lipogenic enzymes (119–122). Moreover, a single intramuscular injection of adeno-associated virus (AAV) encoding leptin into ob/ob mice was able to achieve a long-term normalization of metabolic abnormalities, such as impaired glucose tolerance, insulin resistance, hyperglycemia, and lethargy (123). In instances where no leptin receptor defects are detected, leptin gene transfer may prove beneficial, at least to prevent or delay the onset of obesity as a preventative measure against obesity-induced insulin resistance, which precedes type 2 diabetes. Leptin, acting at the level of the hypothalamus, controls a very complex satiety system and, in this regard, leptin expression could be targeted to tissues in which feedback to the hypothalamus and the pituitary occurs in response to feeding.

The observation that the ratio of leptin in the cerebrospinal fluid to serum in obese individuals is higher compared to normal controls suggests that most

obese individuals do not have defects in the leptin gene and are resistant to its effects either because of a leptin receptor or leptin transport defect (124). These data indicate that alternative strategies aimed at bypassing the hypothalamic targets of leptin thought to be resistant to leptin are necessary and, in this regard, a better objective could be the reestablishment of insulin sensitivity in obese individuals. Identically, such an objective is desired in nonobese insulin-resistant individuals.

Reestablishment of Insulin Sensitivity

Insulin insensitivity is one of the main characteristics of type 2 diabetics and is associated with increased β cell insulin secretion along with hyperinsulinemia. Eventually, β cell exhaustion is thought to occur, which results in overt hyperglycemia. Consequently, insulin resistance can be thought of as two impairments whose temporal relationship is not fully clear: peripheral insulin insensitivity with β cell compensation, culminating in β cell exhaustion (125–127). Whatever the temporal order, the reestablishment of peripheral insulin sensitivity is paramount to prevent β cell exhaustion; therefore, strategies aimed at bypassing defects in insulin sensitivity in peripheral tissues (skeletal muscle, adipose, and liver) or to promote glucose uptake and utilization in the periphery must be considered as a first approach in gene therapy strategies.

The major glucose transporter in the periphery is GLUT-4, and studies with transgenic mice indicate that GLUT-4 expression in peripheral tissues could be one way to prevent or ameliorate insulin resistance. Leptin-resistant, diabetic (db/db) mice (whose genetic defect is in the leptin receptor gene), transgenic for the human GLUT-4 transgene, exhibited reduced fasting and nonfasting plasma glucose levels, improved glucose disposal, and insulin-sensitive, glucose-responsive translocation of GLUT-4 to the cell surface (128). In high-fat fed mice where GLUT-4 expression was driven by the fatty acid binding protein aP2 promoter, an adipocyte-specific promoter, a slight improvement in glucose tolerance and increased transport in adipocytes and adipocyte hyperplasia was observed. That the improvement of glucose tolerance was not major was thought to be due to the absence of the GLUT-4 transgene in skeletal muscle, the major tissue of glucose utilization (129,130). Improvements in glucose tolerance and insulin sensitivity were achieved in transgenic mice where GLUT-4 was driven by skeletal muscle-specific promoters. Hansen et al. (131) demonstrated that glucose

transport into skeletal muscle was significantly increased and insulin-sensitive compared to nontransgenic controls, and, moreover, glycolysis and glycogen synthesis were enhanced in isolated skeletal muscle (131). Additionally, in another transgenic mouse model deficient in GLUT-4, in which GLUT-4 was expressed under the control of the myosin light-chain promoter, the uptake of a synthetic glucose analogue and the actions of insulin were enhanced and restored to normal levels in vivo (132). In a heterozygous GLUT-4 knock-out mouse model, complementation of the gene in skeletal muscle was able to restore insulin sensitivity and prevented a diabetic phenotype (133).

Overexpression of the insulin receptor along with GLUT-4 and insulin receptor-downstream signal transducers such as the insulin receptor substrate (IRS) proteins could be another means of restoring normal insulin sensitivity. The rationale for this derives from studies in homozygous or heterozygous transgenic mice deficient for the insulin receptor, IRS-1 and IRS-2 (134). Muscle-specific insulin receptor knock-out mice are insensitive to insulin in muscle but not in liver or adipose, whereas β cell-specific insulin receptor knock-out mice exhibit an impaired response to glucose, yet are sensitive to insulin in the periphery. However, IRS-1 and IRS-2 knock-out mice are hyperinsulinemic with a mild to severe diabetes where the insulin insensitivity is at the level of muscle and adipose cells in IRS-1 knock-outs and at the level of liver in IRS-2 knock-outs (134). Ueki et al. (135) demonstrated an almost complete restoration of normal glucose sensitivity in IRS-1-deficient mice injected systemically with an adenovirus encoding IRS-1. Other genes that have been transferred to skeletal muscle include GK using an adenoviral vector in vitro. In that study, an increase in glucose uptake was noted, independently of insulin stimulation, as well as a glucose concentration-dependent accumulation of glucose in skeletal muscle (136). In a follow-up study, the same group demonstrated that adenoviral gene transfer of GK to hepatocytes from Zucker diabetic rats (one rodent model of obesity and insulin resistance) was able to promote normal glucose

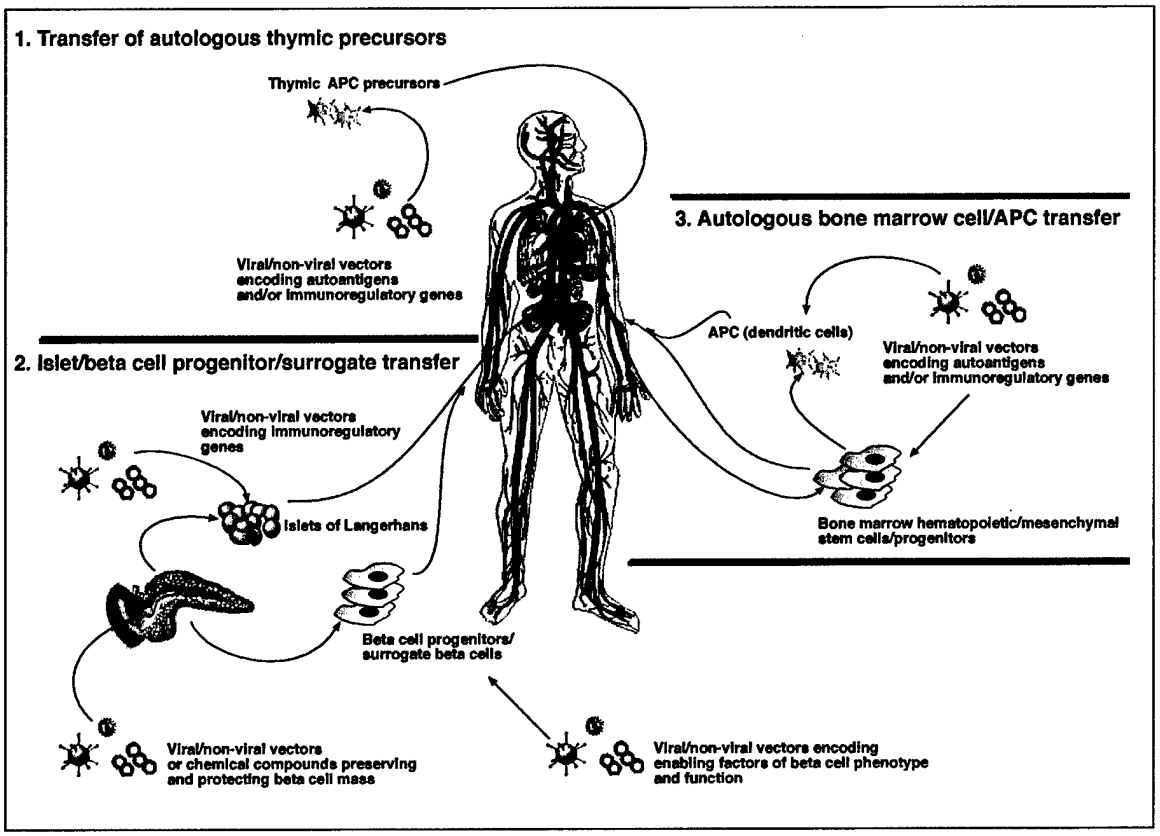


Figure 2. Gene and cell therapy strategies to promote islet allograft/xenograft survival and/or prevent diabetes. Several strategies can be employed alone or in combination: (*i*) gene transfer of immunoregulatory molecules to autologous cells that can modulate immunoregulatory networks (such as dendritic cells); (*ii*) gene transfer of cytoprotective genes to allogeneic or xenogeneic islets that will be subsequently transplanted; (*iii*) protection of β cell mass and function during the isolation phase of islets; (*iv*) gene transfer of factors that promote a β cell phenotype to β cell progenitors or surrogate cells; (*v*) and the direct transfer of vectors encoding immunoregulatory molecules into a susceptible or recent-onset patient.

uptake and conversion to glycogen. More recently, in vivo, transduction of newborn rat skeletal muscle with adenovirus-GK achieved an increase in glucose uptake and ameliorated total body glucose tolerance (137). Finally, Etgen et al. (138) used an adenovirus encoding human protein kinase C-zeta to transduce rat skeletal muscle in vivo. Protein kinase C-zeta is believed to be a constituent of the insulin receptor signaling pathway, and, in that study, the investigators observed enhanced glucose uptake in the skeletal muscle of Zucker rats (138).

Thiazolidinediones are a class of glucose-lowering agents that act to increase insulin sensitivity. Their action is at the level of peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear transcription family of proteins, although PPARγ-independent activities have also been observed (127). Intriguingly, heterozygous knock-out mice for PPARγ do not develop insulin resistance, but, in contrast, display enhanced insulin sensitivity (127). This and other observations suggest that the genetic inhibition of PPARγ activity could prevent insulin resistance, and an approach could be the targeting of dominant-negative variants of PPARγ to insulin-sensitive tissues such as skeletal muscle or adipose.

The activity of cytokines on insulin resistance has been well documented. Tumor necrosis factor-α (TNFα) in particular induces insulin resistance in vitro and in vivo. Cheung et al. (139) demonstrated that systemic gene transfer of a soluble TNF receptor-Fc transgene using an adenoviral vector in Zucker rats enhanced the peripheral insulin sensitivity and suppressed hepatic glucose output (139). It has been suggested that other cytokines such as interleukin (IL)-1β and IL-6 might be involved in insulin resistance, and targeting their binding to receptors using soluble receptor or antagonist decoy strategy might be beneficial.

Insulin Replacement

Where peripheral insulin sensitivity is normal and the defect lies at the level of the β cell, β cell augmentation or replacement strategies may overcome the endogenous defect(s). An approach could be the transplantation of allogeneic islets or the engineering of surrogate β cells. Additionally, the identification of β stem or progenitor cells is also an area that is promising (140–145), especially in light of recent discoveries that bone marrow-derived cells can be made to differentiate into neuronal and muscular cells (146). In the event that the donor is allogeneic, some degree of immunosuppression will be required, and approaches for these strategies have been discussed in an earlier section.

EDUCATING THE IMMUNE SYSTEM

Type 1 diabetes mellitus is perhaps one of several autoimmune diseases where it may be possible to induce tolerance. The autoimmunity is directed against β cells, which suggests that one or more molecules expressed by β cells may be employed to tame the immune system. There are some lines of evidence that support the concept that insulin may be an important β cell protein to which developing T cells failed to be educated. First, are the genetic data, where the insulin locus has been linked to type 1 diabetes (147–151). The second line of evidence in support of insulin as an important autoantigen is the demonstration of circulating autoantibodies and, more recently, an insulin-peptide-reactive cytotoxic T cell clone that was isolated from the islet infiltrate of young NOD mice (152, 153). While insulin remains a strong contender, there is very good evidence in support of other putative antigens such as glutamic acid decarboxylase (GAD) and IA-2 (1,2). These other proteins, however, are expressed in a variety of other nonendocrine cells and

tissues, and it is unclear how they may specifically contribute in type 1 diabetes autoreactivity (154).

If, indeed, type 1 diabetes is the result of a failure to delete lymphocytes in the thymus that would normally recognize β cell antigens, then it is reasonable to propose that thymic over-expression of putative autoantigens in early life, before the onset of type 1 diabetes in individuals deemed at high risk, could prevent the disease by genetic screening criteria. This line of reasoning was initially adopted for studying diabetes in the NOD mouse as well as in the BioBreeding (BB) rat, two rodent models whose diabetes-related immunopathology is considered to be quite similar to that in humans. In NOD mice, the expression of a proinsulin transgene controlled by the MHC class II promoter, resulting in the intrathymic expression of insulin, was able to prevent diabetes (155). This outcome was also achieved in BB rats and in young NOD mice by the intrathymic injection of islet extracts (156–160). In these studies, Posselt et al. (156–158) suggested that tolerance occurred as a consequence of the deletion of islet-reactive thymocytes. The intrathymic injection of insulin B-chain or the 65-kDa variant of GAD into young NOD mice was also able to suppress type 1 diabetes onset (161). However, it is unclear whether this approach will work in post-pubescent humans because of a lack of knowledge concerning thymic delivery. More important is the persistence of the vector for the entire lifetime of the individual and the risks associated with anti-vector response in vivo, depending on the choice of vector, although recent results suggest that thymic gene delivery using adenoviral vectors is possible and can also result in tolerance to the virus-encoded antigens (162).

A second possibility could be to identify the precursors to thymic antigen-presenting cells and to expand them ex vivo concurrent with engineering them to express one or more autoantigens using several gene delivery vectors. Upon introduction into the patient, these cells are expected to migrate to the thymus to express the autoantigen(s). The caveat to this approach is that this cell therapy will be patient-specific and require the isolation of bone marrow cells from which thymic antigen-presenting cell (APC) precursors could be propagated. There is encouraging evidence that this can be clinically feasible. A third and exciting possibilty is to take advantage of peripherally derived APCs that can be engineered to possess tolerogenic properties, acting peripherally and eliminating or silencing circulating autoreactive T cells. In fact, we and others have demonstrated that this approach can facilitate allogeneic cardiac and islet allograft survival, and a minor modification of this approach could be applied to tolerance to autoantigens (163–166).

Full activation of T lymphocytes requires a second signal in addition to the interaction between the T cell receptor (TCR) and the peptide/major histocompatibility complex (MHC) class II complex on an APC (13). This second signal, acting through the CD28 molecule at the surface of T lymphocytes, can be provided by the B7 molecules, B7-1 (CD80) and B7-2 (CD86), which are expressed at the surface of APCs. Blockade of CD28-B7 interaction leads to T cell anergy or apoptosis (167). The fusion, CTLA-4Ig, effectively acts as a decoy for B7 molecules and prevents the B7-CD28 interaction. Several studies illustrated in Table 3 demonstrate the feasibility of the gene transfer of this agent to prevent and modulate type 1 diabetes (168–178).

THE CHOICE OF DENDRITIC CELLS AS EDUCATORS

Although considered potent immunostimulators, dendritic cells (DCs) have recently been shown to possess tolerogenic characteristics under defined conditions. DC tolerogenicity,

manifested as the suppression of T cell activation, has been documented in tumor-, allo-, and auto-immunity (179). The conditions that can yield tolerogenic DCs include ultraviolet irradiation and exposure to CTLA-4Ig, transforming growth factor (TGF)β, or IL-10 (180). How a tolerogenic DC acts to suppress immunoreactivity is not completely understood, but it may involve anergy, a shift to TH2-type responses, apoptosis, or the induction of regulatory cells (179). Myeloid DCs have been genetically modified using adenoviral and retroviral vectors encoding CTLA-4Ig, TGFβ, and IL-10 (180). CTLA-4Ig-expressing DC significantly prolong allograft survival, can induce alloantigen-specific T cell hyporesponsiveness, and display enhanced survival in nonimmunosuppressed, allogeneic hosts (180). The in vivo presentation of alloantigens by donor or recipient DCs in the absence of co-stimulation, along with the local production of immunosuppressive molecules such as TGFβ, could likely promote the inhibition of anti-donor reactivity and promote tolerance induction with no major systemic immunosuppression. DCs engineered to express vIL-10 following retroviral gene transfer produce high levels of vIL-10 in vitro, exhibit marked reduction in cell surface MHC and co-stimulatory molecule expression, decrease T cell allostimulation, and promote the induction of T cell hyporesponsiveness (181). Genetically engineered DCs may be used to prevent islet allograft rejection because they are able to manipulate anti-donor and/or autoantigen immunoreactivity. Alternatively, if recent observations that show islet-specific molecule gene expression in peripheral lymphoid organs can be confirmed in APCs (182) such as bone marrow-derived DCs (unpublished observations), one can envision infusing engineered DCs that lack co-stimulatory capability but that express islet-specific genes (e.g., GAD65 or insulin) into prediabetic or early-onset diabetic patients, with the objective of inducing autoantigen-specific tolerance.

A related approach can be similar to that described by Zhang et al. (183), who have engineered DCs to express Fas ligand as a means of inducing tolerance (183). Peripheral blood mononuclear cells can be cultured ex vivo, and DCs can be propagated using a cytokine cocktail (184). These DCs, or their monocyte precursors, could be engineered with several different gene delivery vehicles, each of which alone or as a multi-cistronic construct could encode putative autotantigens along with proteins that can induce the apoptosis of T cells with TCR that recognize the over-expressed antigen peptides or could silence autoreactive T cells. One can envision, for example, DCs expressing GAD and Fas ligand or CTLA-4Ig. In the first instance, the T cells with TCR specific for GAD epitopes, upon reacting with the DCs, will encounter Fas ligand, and, as activated T cells expressing Fas, will be induced to undergo apoptosis. In the other instance, GAD-reactive T cells will be unable to receive the CD80, CD86 co-stimulatory signal, because of the CTLA-4Ig produced by the DCs and will therefore be unable to proliferate. Indeed, studies in allogeneic transplant models have demonstrated that DCs that are engineered to express TGFβ or CTLA-4Ig can induce donor-specific hyporeactivity in vitro and can significantly prolong allogeneic heart transplants when injected in vivo (180,185). More recently, DCs have been treated ex vivo with oligodeoxyribonucleotide decoys to nuclear factor κB (NF-κB), an important maturational transcriptional mediator in DCs and, when injected into an allogeneic host, were able to prolong the survival of an allogeneic heart (166). It is likely that other transcriptional pathways in APCs could be exploited by decoy nucleotide strategies to present autoantigen in the absence of co-stimulatory signals or in the presence of death ligands to silence or kill autoreactive T cells.

IMMUNOREGULATORY VACCINES

Several studies have shown that the injection of DNA plasmid vectors encoding immunosuppressive genes, such as TGFβ, and a soluble interferon (IFN)γ receptor into the muscle of NOD mice can reduce the incidence of

type 1 diabetes (186–188). The mechanism for protection is unclear but may involve the suppression of maturation of APCs in the periphery and the suppression of autoreactive T cell activity before and during insulitis. Furthermore, the incomplete penetrance of the protection could very well be due to the nature of the plasmid DNA vector and its low persistence, especially at the sites of injection. Another critical factor that may have influenced this exciting approach is the nature of the cells that take up the plasmid vector. It is possible that the immunosuppressive transgene was expressed in migratory APCs at the site of injection of the mice in which protection was achieved, which then could deliver the immunosuppressive protein to peripheral lymphoid organs. In the animals in which no protection was achieved, the vector may have been taken up by other cells such as skeletal muscle and fibroblasts, which, by their nonmigratory character, confined the expression of the immunosuppressive transgene locally at the site of injection. To enhance this approach, one could target vectors to APCs either by pseudotyping with envelopes engineered to possess ligands for APC-specific receptors or in cationic formulations into which these APC-specific proteins are conjugated. Bioballistic gene delivery may offer advantages for this approach over injection, as several studies indicate that Langerhans cells and migratory APCs are more efficiently engineered to express a transgene by gene gun delivery (189,190). Finally, in theory, one could also isolate Langerhans cell progenitors from a patient, expand them ex vivo, engineer them in a manner similar to that described above (i.e., multicistronic vectors encoding autoantigen, along with immunoregulatory gene products) and introduce them subcutaneously, where they will migrate to the peripheral lymph nodes to engage autoreactive T cells.

While manipulating APCs to express autoantigens in a tolerogenic context may be one means of promoting tolerance, an alternative method could involve the engineering of thymic APCs to express human leukocyte antigen (HLA) alleles that are not associated with type I diabetes. The expression of HLA DQ and DR alleles conferring susceptibility, for example, could be suppressed by antisense approaches, followed by the supplementation of the thymus with thymic APCs, engineered ex vivo to express nondiabetogenic class II HLA alleles. The rationale for this approach lies in the observations of Singer et al. (191), who have demonstrated that type 1 diabetes was prevented in transgenic NOD mice expressing a nondiabetogenic MHC class II molecule in place of the diabetogenic I-Ag7. We encapsulate all the approaches reviewed and proposed here in the cartoon in Figure 2.

GENES, CELLS, OR BOTH?

It is premature to make conclusive statements about the future clinical utility of gene therapy for disorders of glucose metabolim, especially diabetes mellitus. Despite the current priority shift from gene transfer to stem cells, more questions and concerns have been raised regarding stem cells compared to gene therapy approaches. In fact, a very recent report appears to refute many pivotal discoveries of putative insulin-producing stem cells (192) and outlines several critical tests that this area of investigation must pass before further consideration. Gene therapy, on the other hand, has passed the conceptual hurdles and has demonstrated strong potential for clinical translation, either as a means of facilitating islet transplantation and/or educating the immune system to accept allogeneic cells and tissues. More emphasis should be placed on the translational research in this realm, with a crucial evaluation of multigenic approaches in low-immunogenicity vectors. Ultimately, we predict that ex vivo engineering of cells will yield the first successful translation of promising animal studies into the clinic.

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In reply

I thank Dr Willoughby for his comments and careful reading of our article.[1] We did our best to control for multiple confounding variables. However, we concur with Dr Willoughby that there may have been other variables, such as precipitous delivery, that could not be controlled for and thus may have accounted for some of the variability in cystic periventricular leukomalacia (PVL). We agree that longer-term follow-up, looking for outcomes such as cerebral palsy, would be ideal. However, we disagree with Dr Willoughby's sentiment that cystic echolucencies are clinically unimportant. Cystic PVL has been associated with a substantial risk of later CP or motor delay.[2-4] Thus, although some infants with cystic PVL may ultimately be free of later motor disability, cystic PVL remains a clinically significant marker of early brain injury. We would like to reemphasize that our data show an association between antenatal antibiotics and reduced risk of later cystic PVL, and not proof of protection. We agree with Dr Willoughby that our data do not provide for an expanded indication for antimicrobial use in pregnancy and that randomized trials are needed prior to any initiation of antenatal antibiotic treatment strategies to decrease the occurrence of neonatal brain injury.

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Need for Genetic Education for Type 1 Diabetes

We read with interest a recent article that appeared in the ARCHIVES regarding the ethics of predictive genetic screening for type 1 diabetes (T1D).[1] Dr Ross nicely reviewed the status of newborn genetic screening for T1D, which is beginning to be offered at a statewide level in the United States. The primary purpose of newborn genetic screening is the identification of high-risk infants. Currently, more than 90% of parents consent. Babies at high risk (~2%-10%) are recruited into natural history studies. The American Diabetes Association Position Statement indicates that genetic screening for T1D outside the context of research is not warranted.[2] Dr Ross addressed the ethics of newborn genetic screening for a nonpreventable disorder, such as T1D. Her concerns included the psychosocial risks of predictive testing, the false assurance for children not considered to be at high risk, and proper informed consent.

We share Dr Ross' concerns, and describe here our approach for addressing these issues, which were discussed at a National Institutes of Health–Sponsored Conference on Behavioral Science Research in Diabetes (November 18-19, 1999; Bethesda, Md). This meeting emphasized the need to provide accurate risk information, maximize the benefits of determining risk status, minimize the distress during risk notification, and educate children, families, and health professionals regarding genetic testing for T1D.

To begin to address these needs, we are developing Internet-based programs titled Genetic Information for Testing (GIFT) for T1D to serve as "decision aids" to improve the consent process.[1] Programs are targeted for families who are considering genetic testing for T1D. These contain modules on genetics, treatment, risks and benefits of testing, and genetic counseling for T1D. A personalized risk algorithm based on genetic/environmental risk factors is also included. Psychosocial measures related to the Health Belief Model[3] have been incorporated for evaluation purposes. A separate program is intended for health professionals who offer genetic testing for T1D. This program is for educational purposes (with continuing education credits), and will enhance the provision of "more active parental consent."[1]

Although our focus is on T1D, genetic testing is fast becoming a significant part of life and medical practice. Thus, knowledge of genetic and environmental determinants of disease must be available to consumers and practitioners, and is it essential for developing prevention strategies and establishing appropriate health policy. It is anticipated that our programs will assist with the trans-

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lation of scientific information for T1D and possibly other disorders, from the laboratory to the community.

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In reply

I thank Dr Dorman and colleagues for sharing their thoughtful project to educate families and clinicians about genetic testing for type 1 diabetes. It is important that the project will focus on families with an affected biological relative, as their "high-risk" status changes the risk-benefit of genetic testing at this time when no preventive measures exist.

I agree with Dr Dorman and colleagues that the development of decision aids will be important for the translation of scientific information on type 1 diabetes from the bench to the community. Policy guidelines must be developed to ensure that the introduction of testing, both for research and clinical purposes, minimizes risks.[1] One consideration must be the appropriate community for such testing when no therapies exist. Children are a vulnerable population; infants, more so.[2-4] Type 1 diabetes is the most common metabolic condition of childhood and, therefore, the inclusion of children in this research is important. In my article, however, I questioned whether the general newborn population is the appropriate community for study if there are plans to disclose risk information to families. I concluded that it was not.[1] In another article, I examine the ethics of prediction and prevention research in type 1 diabetes beyond the newborn period and how such research should be evaluated by institutional review boards given current federal regulations.[5] The tools being developed by Dr Dorman and colleagues will help make type 1 diabetes research in high-risk families pass ethical review.

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ELSEVIER

HLA Class II DRB High Resolution Genotyping by Pyrosequencing: Comparison of Group Specific PCR and Pyrosequencing Primers

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ABSTRACT: Sequencing of alleles of the highly polymorphic, multiple loci HLA-DRB gene family was performed by pyrosequencing using purified DNA from the 11th International Histocompatibility Workshop human lymphoblastiod cell lines as well as genomic DNA isolated from blood samples obtained from healthy adult volunteers. Genomic DNA was prepared from donors whose blood had been stored either frozen or as dried blood spots. Pyrosequence-based typing was optimized for identifying alleles of the HLA-DRB1, -3, -4, and -5 genes. The procedure should be applicable to other HLA loci including the class I genes HLA-A and -B that, along with HLA-DRB, are crucial for histocompatibility matching of tissue antigens during transplantation. Computer simulation of pyrosequencing data suggest that alleles of HLA-DRB1, -3, -4, and -5 were readily identifiable by pyrosequencing as were their heterozygous allelic combinations. Pyrosequencing primers were designed to specifically sequence HLA loci of interest even in a background of other amplified, closely related sequences such as alleles of the pseudogene HLA-DRB6, -7, -8, and -9. Polymorphic residues of HLA-DRB genes were identified within each pyrosequencing reaction, obtained by 50 to 70 nucleotide read lengths. Heterozygous allelic combinations of HLA genes were analyzed and compared successfully to genotyping of alleles by sequence-specific oligonucleotide probe hybridization as well as allele specific polymerase chain reaction protocols. Pyrosequence-based typing is compatible with genotyping of allelic combinations expected from heterozygous individuals, resulting in nucleotide resolution of the highly polymorphic HLA system. Using a single pyrosequence instrument, complete typing of HLA-DRB genes can be performed daily on hundreds of individuals for high resolution histocompatibility genotyping studies. *Human Immunology 65, 163–174 (2004).* © American Society for Histocompatibility and Immunogenetics, 2004. Published by Elsevier Inc.

KEYWORDS: histocompatibility; transplantation; autoimmune disease; single nucleotide polymorphism

ABBREVIATIONS

DBS	dried blood spot
EST	expressed sequence tag
HLA	human leukocyte antigen
PSBT	pryosequence-based typing
SBT	sequence-based typing
SSOP	sequence-specific oligonucleotide probe
SNP	single nucleotide polymorphism
SSB	single-stranded binding protein

INTRODUCTION

Accurate human leukocyte antigen (HLA) genotyping is essential for matching of tissue during bone marrow as well as solid organ transplantation. Analysis of genomic DNA obtained from families plagued by high occurrence of autoimmune disease have provided critical information linking the risk of developing autoimmune disorders to certain HLA alleles. For example, nucleotide polymorphisms at HLA class II loci have been correlated with susceptibility to a variety of autoimmune diseases, such as type 1 (insulin dependent) diabetes, celiac disease, and rheumatoid arthritis [1, 2]. A wide body of

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evidence has linked specific polymorphisms of the HLA-DRB and DQB1 loci, such as DR3 or DR4, along with inheritance of non-Asp-57 residue in the DQB1 gene product, as being the strongest indicators of susceptibility for type 1 diabetes [3–13]. Meanwhile, the HLA DR3-DQ2 haplotype has been associated with celiac disease [14–16], and inheritance of HLA-DRB1*0401 has been correlated with increased risk of developing severe forms of rheumatoid arthritis [17].

Development of rapid and cost effective strategies for screening of the highly polymorphic HLA loci for alleles associated with autoimmune disease is important for risk evaluation of genetically susceptible individuals. The method of pyrosequence-based typing (PSBT) provided high resolution genotyping of alleles of the HLA loci, unambiguously identifying HLA-DQB1 alleles obtained from polymerase chain reaction (PCR) amplified samples of genomic DNA from cell lines as well as DNA samples isolated from human donors [18–20]. PSBT succeeded in determining the identity of polymorphisms encoding amino acid substitutions at specific, disease-correlated codons, such as the presence of a non-Asp residue at codon 57 of HLA-DQB1, as well as in obtaining high resolution sequence data leading to unequivocal allelic genotyping. Genotyping of individual alleles, along with haplotype information, improves the accuracy of evaluating an individual's risk for developing various HLA-linked autoimmune diseases [21–24]. Moreover, when applied to HLA-DRB and the class I genes HLA-A and -B, PSBT will be useful for high resolution typing of the HLA alleles required during successful kidney [25, 26] and bone marrow transplantation [27, 28].

Pyrosequencing was originally designed for analyzing expressed sequence tags (ESTs) in which short (roughly ten nucleotide) stretches of DNA are sequenced. The pyrosequencing method [29–32] exploits a multienzyme process combining the activities of DNA polymerase, ATP sulfurylase, and apyrase, in the conversion of inorganic pyrophosphate (a product normally generated upon incorporation of a nucleotide into a nascent DNA chain) into ATP and subsequently utilized by luciferase in the breakdown of its substrate resulting in the production of light. Thus, generation of light indicates incorporation of a particular nucleotide. Conceptually similar to the process of sequence-specific oligonucleotide probe (SSOP) based HLA genotyping, but by single nucleotide hybridization, pyrosequencing is performed by reiteration of steps in which an individual dNTP is added and tested sequentially for incorporation into the nascent nucleotide chain, well suited to analysis of polymorphic DNA. Pyrosequencing has been readily able to read DNA sequences of up to 70 nucleotides [18], and sequences as long as 150 residues have been reported [33, 34]. An advantage over other sequencing technologies is that single nucleotide dispensation during pyrosequencing results in out-of-phase sequencing of DNA strands allowing sequence information to be obtained from each allele independently [34]. The methodology allows resolution of allelic combinations that are difficult to distinguish by conventional sequencing approaches [18–20, 35–37]. Combined with the development of HLA allele specific pyrosequencing primers, PSBT greatly improved high resolution HLA genotyping when more than one gene is PCR amplified, such as occurs when analyzing the potentially multiple loci HLA-DRB family in which a variety of genes exhibiting high sequence conservation are amplified simultaneously.

Our study was intended to expand on the development of PSBT for the identification of HLA alleles in clinical samples [18] while accomplishing the genotyping of the complex multi-loci HLA-DRB system. HLA-DRB represented the added challenge of typing polymorphic DNA from as many as four expressed loci, along with the requirement of obtaining sequence from stretches of roughly 300 nucleotides containing as many as 121 polymorphic residues, whose identity is essential during evaluation of susceptibility to autoimmune disease as well as for successful matching of recipient and donor tissue during transplantation. The HLA-DRB system consists of not only multiple expressed loci but a variety of pseudogenes as well. Samples of genomic DNA prepared from cell lines homozygous for HLA loci on human chromosome 6 made available at the 11th International Histocompatibility Workshop [38] and from healthy volunteers were selected for genotype analysis. Pyrosequencing was used to identify alleles at the highly polymorphic exon 2 region of the HLA-DRB expressed genes. PSBT allowed routine sequencing of 50 to 70 nucleotide long read lengths of each sample in a 96-well sample tray within roughly an hour's time, potentially yielding 768 sequence reactions in an 8-hour shift from a single instrument. Accuracy, improved resolution of allelic pairs, and throughput make PSBT an important addition to methods available for identification of known markers in studies of the genetic association of certain HLA alleles with the likelihood of developing autoimmune disease as well as during clinical genotyping of patients and donors for histocompatibility matching during transplantation.

MATERIALS AND METHODS

Materials

Oligonucleotides, listed in Table 1, were purchased from Qiagen-Operon Inc. (Alameda, CA, USA) or Integrated DNA Technologies (Coralville, IA, USA). Biotinylated oligonucleotides were high performance liquid chromotography purified. Genomic DNA samples were ob-

TABLE 1 Oligonuclcotide primers used for PCR and DNA sequencing

Name	Sequence (written 5′ to 3′)	Comment
PCR primers for HLA-DRB exon 2		
SR81	Biotin-CCGCTGCACTGTGAAGCTCT	DRBampB biotinylated reverse primer
SR81a	CCGCTGCACTGTGAAGCTCT	DRBampB reverse primer
SR88	AATCCCCACAGCACGTTTCCTG	DRBampA(C) forward primer
SR89	AATCCCCACAGCACGTTTCTTG	DRBampA(T) forward primer
SR147	GTTTCTTGGAGCAGGTTAAAC	DRB1 forward primer for groups DR4
Pyrosequencing primers for HLA-DRB exon 2		
SR83	TCAATGGGAC	Residues 40–49
SR84	TCAATGGGAT	Residues 40–49
SR85	TCAACGGGAC	Residues 40–49
SR86	GGGCGGCCT	Residues 147–155
SR87	GGAACAGCCA	Residues 169–178
SR131	TGGAGCAGGTTA	Residues 10–21; DRB1*04 specific in DRB1*07, DRB4 background

Primers SR81, SR88, SR89, and SR147 were designed following the recommendations of the 11th International Histocompatibility Workshop [38] but with the additional constraint of avoiding formation of self-priming 3′ secondary structure during pyrosequencing. Oligonucleotides SR88 and SR89 were also used during PSBT and initiated DNA sequencing after residue 11 of HLA-DRB exon 2.

Abbreviations: HLA = human leukocyte antigen; PCR = polymerase chain reaction; PSBT = pyrosequence-based typing.

tained from the lymphoblastoid cell lines made available on the occasion of the 11th International Histocompatibility Workshop [38], or from healthy donor volunteers (Table 2). The pyrosequencer was purchased from Pyrosequencing, AB (Uppsala, Sweden). Single-stranded binding protein (SSB) was purchased from USB Corp. (Cleveland, OH, USA). All other chemicals and reagents were of the highest quality obtainable.

TABLE 2 Identity of DRB alleles in clones and cell lines sequenced in this study

Samples	PSBT identified alleles
Clones	
	DRB1*010101
	DRB1*130201
	DRB1*1402
	DRB3*0101
	DRB3*020201
	DRB3*030101
	DRB6*0101
	DRB7*010101
Lymphoblastoid cell line	
CB6B	DRB1*1301;DRB3*0202
DKB	DRB1*090102;DRB4*0103
MGAR	DRB1*1501;DRB5*0101;DRB6*0201
PGF	DRB1*150101;DRB5*010101;DRB6*0201
RAJI	DRB1*030101;DRB1*100101
SPO010	DRB1*1101;DRB3*0202
WT47	DRB1*1302;DRB3*0301
YT	DRB1*0405;DRB4*0101

Lymphoblast cell lines made available at the 11th International Histocompatibility Workshop [38]. Genotypes of lymphoblastoid cell lines are from the IMGT/HLA database [42].

Whole Blood Samples

Whole blood was drawn by vein puncture into vacutainer-ACD tubes (Becton-Dickinson Inc., Franklin Lakes, NJ, USA). Samples were split into three pools and were either processed immediately for purification of genomic DNA, stored frozen at −80 °C, or spotted onto S&S 903 paper (Schleicher & Schuell Inc., Keene, NH, USA). Blood spots were allowed to dry overnight in a biosafety cabinet prior to transfer to a sealed container for storage at room temperature. Frozen and dried blood spot (DBS) samples were stored at least 1 week prior to DNA preparation.

Preparation of Genomic DNA

Extraction of genomic DNA from fresh and frozen whole blood was performed using a QIAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA, USA) as directed by the manufacturer, typically yielding greater than 0.5 μg of genomic DNA per 75 μl of whole blood. Purification of genomic DNA from DBS samples was preformed as recommended by Caggana *et al.* [39]. Briefly, punches were taken from the DBS using a Wallac Delfia DBS Puncher (Perkin-Elmer Life Sciences Inc., Turku, Finland) equipped with a 1.5-mm punch head. Specimens were punched directly into 96-well tray, one punch per well. Each sample was incubated in 100 μl methanol and air-dried overnight at ambient temperature in a fume hood. After drying, samples were treated with 25 μl distilled water, sealed and heated to 98 °C for 10 minutes. DNA samples were cooled to room temperature (roughly 23 °C) prior to PCR [40].

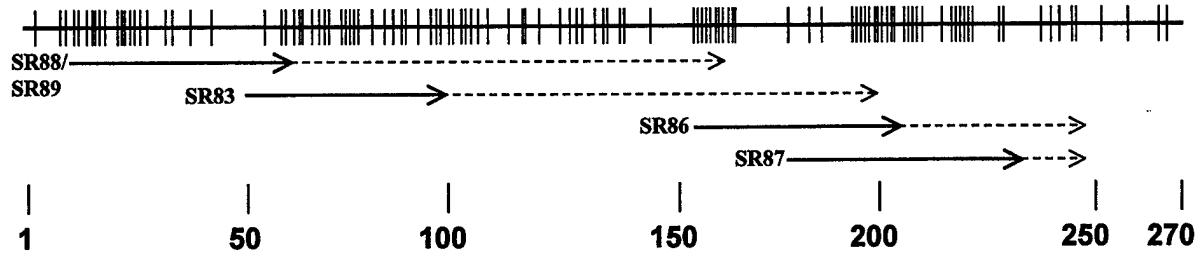


FIGURE 1 Relative locations of HLA-DRB polymorphisms within exon 2 for loci 1–9 and regions sequenced by PSBT. The sites of polymorphic residues are indicated by vertical lines while the locations of regions sequenced by PSBT are indicated by arrows. Solid arrows indicate sequenced regions for 50 nucleotide read lengths, whereas dashed arrows denote regions that would be observed during 150 nucleotide read lengths using methods reported by Gharizadeh *et al.* [33]. Polymorphic sites 5′ of residue 12 and 3′ of residue 246 are unavailable for sequencing as they overlap sites for PCR primer annealing. The nucleotide index numbers are also indicated. Abbreviations: HLA = human leukocyte antigen; PCR = polymerase chain reaction; PSBT = pyrosequenced-based typing.

DNA Cloning

Cloning of HLA-DRB loci was performed using a PCR cloning kit purchased from Qiagen Inc. Amplification of genomic DNA was performed using oligonucleotides SR81a, SR88, and SR89 as primers (Table 1). PCR amplified DNA was ligated into the pDrive cloning vector using EZ competent cells, both provided by Qiagen, Inc. Transformed cells were identified by screening for ampicillin resistance. Initial identification of HLA-DRB locus and allele was determined by sequencing using an ABI 3100 Genetic Analyzer and classified using HLA genotyping analysis software (Applied Biosystems Inc., Foster City, CA, USA).

Polymerase Chain Reaction

The PCR amplification was performed in 50 μl volumes containing Taq buffer (Applied Biosystems, Inc.), 2-mM $MgCl_2$, 0.2 μM each dNTP, 0.2 μM forward and biotinylated reverse primers, 1 unit Taq polymerase, and 5 μl purified genomic DNA (roughly 200 to 500 ng DNA) or using DNA extracted from a single 1.5-mm DBS punch treated with methanol as described previously. Amplification included a 96 °C incubation for 3 minutes followed by 32 cycles at 96 °C, 55 °C, and 72 °C incubated for 30 seconds at each step. PCR cycling was followed by a final 5-minute incubation at 72 °C. Samples were then stored at −20 °C or 4 °C prior to pyrosequencing. Amplification of DRB loci 1–9 were performed using primer sets SR81, SR88, and SR89 although group specific amplification of DR4 was performed with SR81 and SR147 (Table 1).

Pyrosequencing-Based Typing

Pyrosequence reactions were performed using reagents provided with the PSQ 96 Sample Preparation kit and the PSQ 96 SQA Reagent kit purchased from Pyrosequencing, AB. Briefly, 20 μl to 40 μl samples of amplified DNA from the PCR reaction mixture were mixed with 4-μl streptavidin coated beads purchased from Amersham-Pharmacia Biotech, Inc. (Piscataway, NJ, USA), denatured to obtain single-stranded amplicons, and prepared for pyrosequencing as recommended by the manufacturer. The appropriate pyrosequencing primer was added to each well in a volume of 5 μl using a 3-μM stock solution. Primer annealing was performed by incubating the samples at 80 °C for 2 minutes and allowed to cool for 5 minutes at room temperature prior to pyrosequencing. When added, single-stranded binding protein was present at 4 μg per pyrosequencing reaction. A detailed description of the pyrosequencing reaction conditions has been recently published by Gharizadeh *et al.* [33]. Pyrosequence data were quantified and background corrected using PSQ 96MA version 2.0.2 software (Pyrosequencing, AB).

HLA Genotyping Kits

Validation of HLA typing of volunteer samples was performed using RELI SSO HLA-DRB1 kits purchased from Dynal Biotech, Inc. (Lake Success, NY, USA). Samples were prepared as recommended by the manufacturer. Determination of HLA-DRB1 genotype was performed by comparison of hybridization data with the expected outcomes for known alleles provided by Dynal Biotech, Inc.

Computer Software

The PSBT simulation and analysis software (CP and SR, unpublished data) was developed in house using Perl v5.6.1 provided by ActiveState Tool Corp. (Vancouver, Canada) under the General Public License. Simulation software was used to estimate the peak signal values expected during pyrosequencing followed by comparison

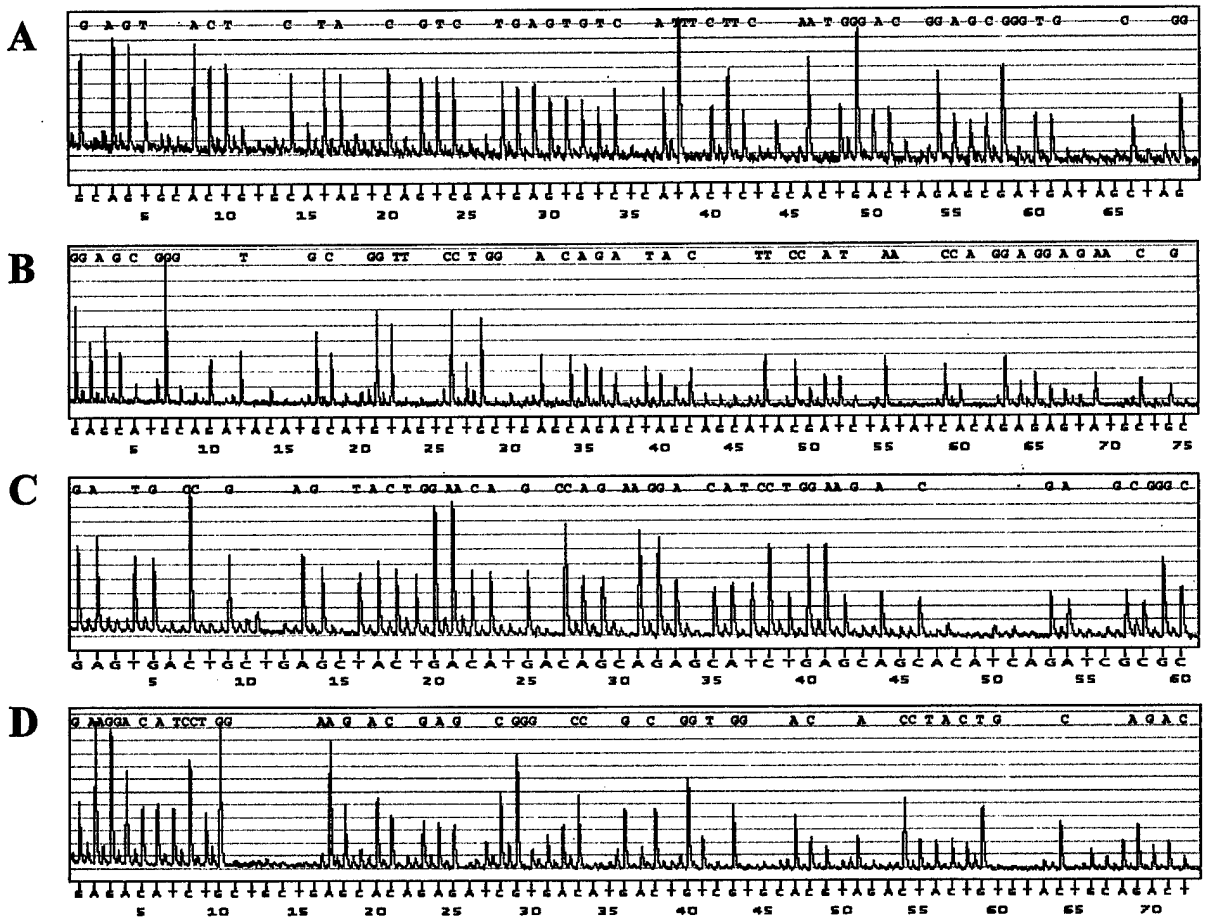


FIGURE 2 Pyrosequencing of polymerase chain reaction product from cloned HLA-DRB1*1302. Pyrogram obtained using sequencing primer SR88/SR89 (panel A), SR83 (panel B), SR86 (panel C), and SR87 (panel D). The expected sequence is indicated near the top of each panel.

of nucleotide dispensation orders and allelic sequence data. PSBT simulation software also compared simulated peak height data for each possible heterozygous allelic combination in order to distinguish unique and ambiguous allelic combinations. Analysis of pyrosequencing data was performed using PSBT analysis software and compared the observed pyrosequencing signals with that anticipated by the simulation software for each potential heterozygous combination of alleles. The allelic pair most closely matching the observed data was reported. Documentation for Perl can be found at the Perl Homepage (http://www.perl.com).

RESULTS

Pyrosequencing methodology was evaluated as a sequence-based strategy for HLA-DRB genotyping. Previous work has indicated that, in the form of PSBT, the method provided an accurate means for discerning the identity of individual alleles of the HLA-DQB system [18]. Expanding PSBT for the analysis of alleles of the HLA-DRB system involved overcoming the complications associated with accounting for multiple alleles from four expressed loci (DRB1, DRB3, DRB4, and DRB5) as well as minimizing the impact from alleles of the non-expressed DRB pseudogenes (*ie*, DRB6, DRB7, DRB8, and DRB9). Computational analysis of alleles from DRB1-9 loci indicated that sequencing results could be obtained either from priming of DNA synthesis at a few universally conserved sites or by choosing primer sites whose occurrence was unique to a subset of alleles (Table 1). Suitable primers were selected by analysis of sequences from exon 2 of the greater than 400 reported alleles of DRB. Results indicated the presence of at least four convenient sites, shared by all alleles, for testing of primers for PSBT (indicated in Table 1 and Figure 1). Depending on the genetic background, a large number of potential group-specific priming sites can also be identified (data not shown). As illustrated in Figure 1, of the

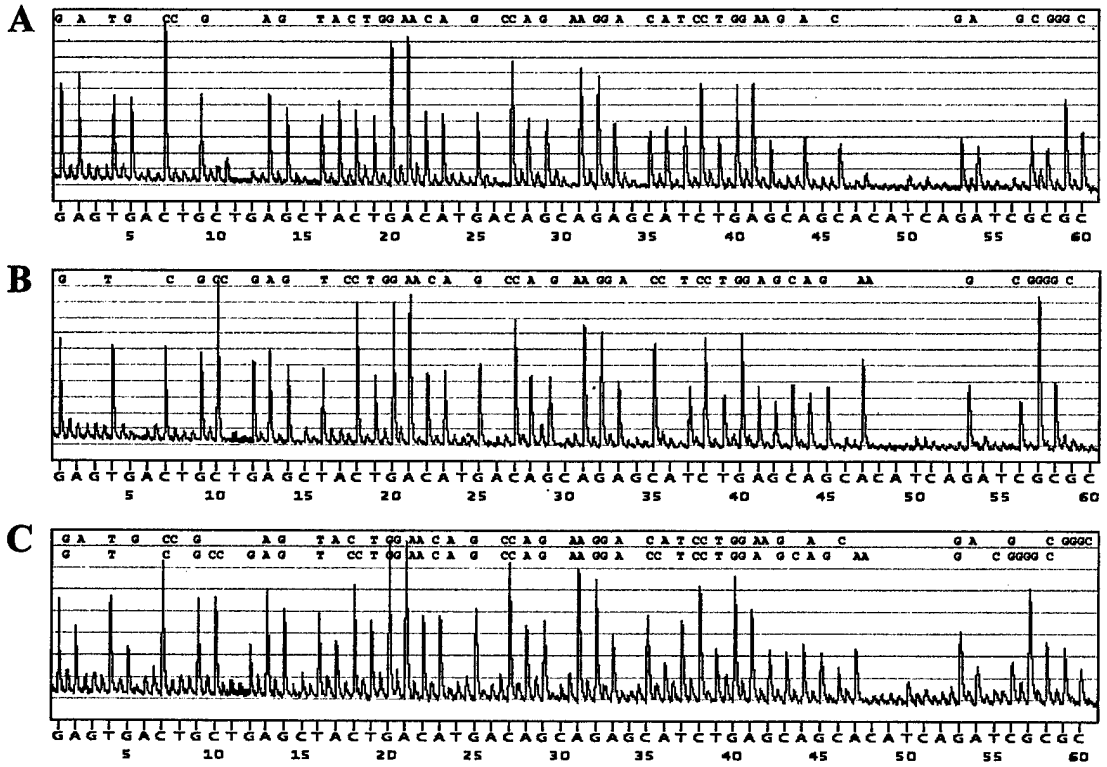


FIGURE 3 Pyrograms of cloned HLA-DRB genes DRB1*1302 and DRB3*0301 and the cell line WT47 HLA-DRB1*1302;DRB3*0301 genomic DNA. Samples were prepared for pyrosequencing from cloned DNA HLA-DRB1*1302 (panel A) and HLA-DRB3*0301 (panel B) or from genomic DNA purified from the homozygous cell line WT47 (panel C). The expected nucleotide sequence for the HLA-DRB alleles for each sample are indicated. Read lengths were at least 50 nucleotides.

114 polymorphic residues contained within exon 2 of DRB, between 81 and 107 can be determined via PSBT using pyrosequencing primers SR88/SR89, SR83, SR86, and SR87 depending on the sequencing read length obtained [18, 33]. Improved read lengths as long as 150 nucleotides have been obtained by optimization of the pyrosequencing reaction conditions [33]. Use of enantiomer pure 2′-deoxyadenosine-5′-O′-(1-thiotriphosphate) Sp-isomer as well as addition of single-stranded binding protein stabilize pyrosequencing signals, thus leading to increased read lengths [33, 41]. Monitoring of the remaining polymorphic sites can be accomplished through selection of additional PSBT primers, when necessary, to allow further evaluation of allele identity.

Pyrosequencing primers optimized for PSBT of HLA-DRB loci were tested using cloned alleles representing various HLA-DRB loci (Table 2). The results obtained from cloned DRB1*1302, for example, are illustrated in Figure 2. The conserved primer set (SR88/SR89, SR83, SR86, and SR87) routinely yielded sequencing information for roughly 50 nucleotide long stretches of exon 2. Nucleotide dispensation orders were chosen to facilitate sequencing of the entire set of HLA-DRB alleles initiated by each of the four PSBT primers. Inspection of the pyrograms indicated that the observed pyrosequencing signal matched the expected values for DRB1*1302 as well as for the other alleles tested (Figure 2 and Table 2). The conserved primer set resulted in sequence data from two sections of DRB exon 2, obtaining 88 and 75 consecutive nucleotides for the regions covered by primer pairs SR88/SR89 through SR83, and SR86 through SR87, respectively. As expected, loss of pyrosequencing signal was observed during nucleotide extension steps, and occurred at a rate of approximately 1%–2% per added residue. Comparison of the observed pyrosequencing signal and allele sequence indicated a positive correlation between the number of nucleotide residues incorporated and pyrosequencing signal. Moreover, the signal from background was constant during the experiment indicating sequencing of a single HLA allele as expected for these samples (Figure 2 and Table 2).

The HLA-DRB genes amplified from genomic DNA have also been analyzed by pyrosequencing (Figure 3). Comparison of pyrograms obtained from cloned DNA HLA alleles DRB1*1302 and DRB3*0301 and genomic

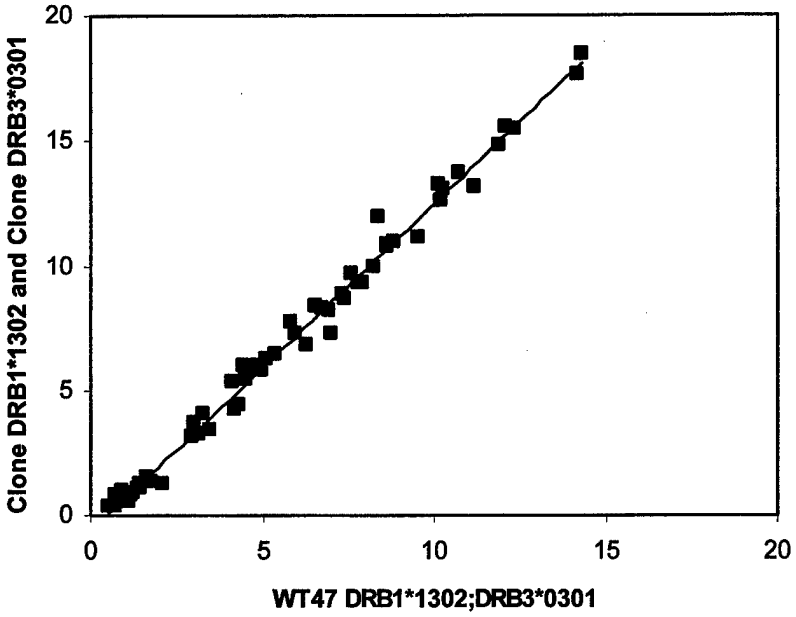


FIGURE 4 Comparison of pyrosequencing signal from the peak heights illustrated in the pyrograms from Figure 3. Peak heights for each nucleotide dispensation were compared. The peak heights from the cell line WT47 DRB1*1302; DRB3*0301 are indicated in the x-axis, whereas the weighted average of the peak heights observed from clones of HLA-DRB1*1302 and HLA-DRB3*0301 are indicated in the y-axis. Linear regression analysis indicated an r^2 value of 0.99.

DNA from WT47 cell line has indicated that genomic DNA containing alleles DRB1*1302;DRB3*0301 can also be successfully analyzed (Figure 3). Pyrograms obtained using the set of universal primers indicated that allele DRB1*1302 specific nucleotide dispensations occurred at events A2, G5, A17, A36, A41, C46, A54, G59, and C60 (Figure 3A), whereas allele DRB3*0301 associated events were at dispensations C10, G12, C43, G45, A47, and C56 (Figure 3B). These same sequencing signals were observed in the data obtained from the WT47 cell line genomic DNA homozygous for alleles HLA-DRB1*1302 and HLA-DRB3*0301 (Figure 3C). The plot, illustrated in Figure 4, also indicated that HLA-DRB1*1302 and DRB3*0301, whether obtained from cloned or from genomic DNA, resulted in identical pyrosequencing signal intensities. Plotted are peak height data from each nucleotide dispensation observed from pyrosequencing of the WT47 cell line (DRB1*1302;DRB3*0301) on the x-axis against the weighted average of the peak height intensities from clones DRB1*1302 and DRB3*0301 on the y-axis (Figure 4). Linear regression analysis indicated a regression coefficient of roughly 0.99. PSBT of HLA-DRB, although matching the expected sequence for these alleles, yielded readily interpreted pyrosequence data whether originating from cloned stocks or from genomic DNA. The data illustrate the additive nature of pyrosequencing when multiple alleles are PCR amplified and sequenced.

The PSBT of HLA-DRB from human blood samples presented the challenge of typing polymorphic DNA from as many as four expressed loci in the presence of, potentially, as many pseudogene loci. Extension of PSBT to simultaneous sequencing of multiple amplified alleles may be problematic due to the inherent noise and variable background associated with prevalent HLA pseudogenes (data not shown). However, accurate knowledge of the expressed HLA-DRB loci is essential for successful matching of recipient and donor tissue during transplantation as well as during evaluation of susceptibility to autoimmune disease. Computer analysis of the HLA-DRB and -DQB sequences, obtained from the IMGT/HLA database [42], have been performed in order to identify suitable sequences for priming pyrosequencing. Group-specific priming sequences were examined for accurate pyrosequencing of HLA-DRB1*04 in the presence of the donor HLA-DRB1*04,*07; DRB4* genotype (Figure 5). As illustrated in Figure 5, group-specific PSBT of HLA-DRB was successfully performed using genomic DNA originating from blood. This material was typed in parallel using commercially available HLA typing SSOP kits (Dynal, Inc.) confirming their HLA-DRB genotype. Experiments focused on using either DRB group specific primers (panel A) or universal PCR primers (panel B) during PCR. Both methods for HLA genotyping analysis were consistent with HLA-DRB1*04,07;DRB4* for this sample (Figure 5). In each

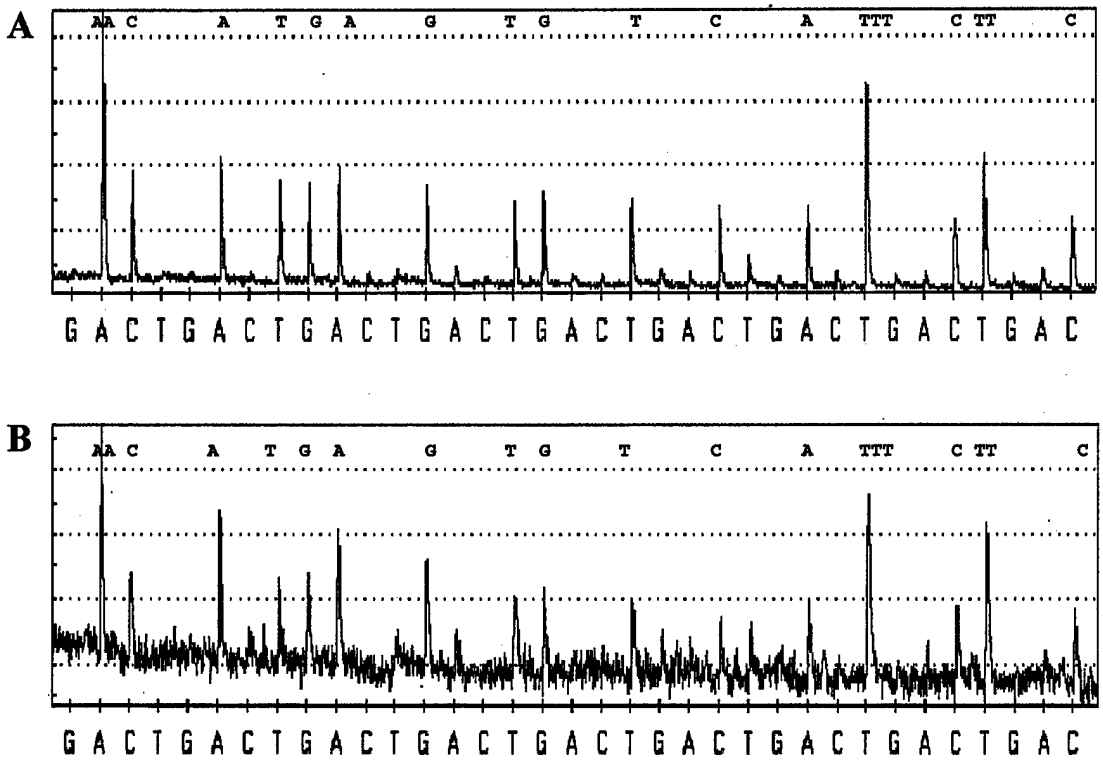


FIGURE 5 Pyrosequence of HLA-DRB. PCR amplified DNA was prepared from human genomic DNA. The HLA-DRB genotype of the donor was DRB1*04, *07 and DRB4* as determined by SSOP genotyping. In each panel pyrosequencing was performed using the DRB1*04 specific sequencing primer SR131 and a cyclic nucleotide dispensation order. Panels illustrate the pyrosequence signals obtained from PCR amplified DNA obtained from either the DR4 group specific PCR primers SR81 and SR147 (panel A) or the universal PCR primers SR81 and SR88/SR89 (panel B). Pyrosequencing conditions were the same except that the pyrosequencing reaction illustrated in the bottom panel included 0.5 μg *E. coli* single-stranded binding protein. The sequence corresponding to the appropriate region of HLA-DRB1*04 is illustrated in each panel. Abbreviations: HLA = human leukocyte antigen; PCR = polymerase chain reaction; SSOP = sequence-specific oligonucleotide probes.

panel the expected HLA-DRB1*04 sequence is indicated. Comparison of the pyrosequence signal intensities observed for the top and bottom panels of Figure 5, are illustrated in the plot depicted in Figure 6, and indicated that either approach successfully resolved the DR4 genotype allele from the HLA-DRB pyrosequence pattern. Exploitation of the methodology is anticipated to allow the generation of PSBT data from these alleles individually, greatly improving our ability to accurately identify the presence of a particular HLA genotype.

DISCUSSION

The PSBT resulted in enhanced allelic resolution relative to other commonly used HLA genotyping methods, such as SSOP and commercially available HLA genotyping kits based on group-specific PCR amplification [18]. PSBT represents an improvement due to the method's ability to exploit single nucleotide addition to the reaction mixture, enabling the collection of allele specific data via out-of-phase sequencing [34]. Moreover, although working with mixed DNA sequences obtained from heterozygous individuals PSBT circumvented the need to isolate individual alleles prior to sequencing, it allowed simplified PCR strategies for preparing genomic DNA for sequence analysis. The combined use of single-stranded binding protein and group-specific PSBT primers resulted in improved read lengths of individual alleles from a mixture of amplified HLA alleles and loci. Technologic advancements, such as automated data acquisition and analysis, are important additions to the screening and characterization of genetic markers. PSBT of class II HLA alleles was performed on prevalent DRB genotypes. Pyrosequencing confirmed the assignment of alleles obtained by SSOP-based typing (Figure 5), and provided additional resolution for genotyping [18, 43].

The PSBT is a convenient tool for analysis of HLA genotypes, providing increased resolution compared with other sequence-based approaches due to its capacity to

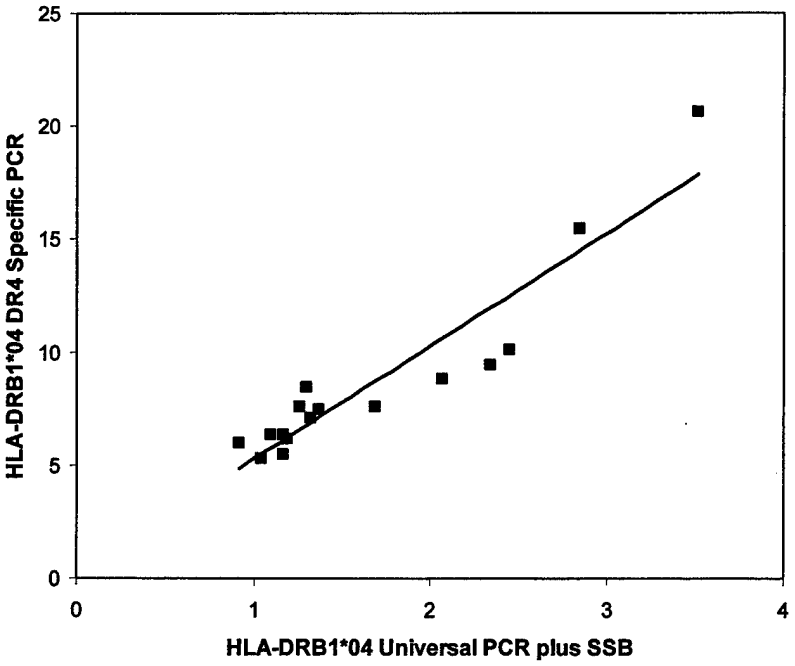


FIGURE 6 Plot of the pyrosequencing signal intensities obtained from panels in Figure 5. The peak heights from pyrosequencing of DRB1*04,*07, and DRB4 when performed as in Figurse 5A and 5B are indicated in x and y axes, respectively. Linear regression analysis indicted a r^2 value of 0.87 and a slope of 5.0. Abbreviations: HLA = human leukocyte antigen; PCR = polymerase chain reaction; SSB = single-stranded binding protein.

enable out-of-phase sequencing [18]. Optimization of PSBT for high resolution HLA typing is accomplished via choice of nucleotide dispensation order as well as by selection of primers for pyrosequencing and PCR. For example, the order of nucleotide dispensation can promote out-of-phase sequencing, greatly influencing the resolution of allelic combinations obtained by PSBT (Figure 7). Cis-trans combinations of sequence motifs of the sort illustrated in Figure 7 indicate the principal cause of ambiguous sequence information when analyzing heterozygous HLA samples using standard techniques (Figure 7A). However, exploitation of out-of-phase sequencing during PSBT provided resolution of these same allelic combinations (Figure 7B). Different nucleotide dispensation orders applied to the same combination of HLA-DRB alleles can yield different pyrosequencing signatures. This directly results in unique PSBT signatures for closely related sequences, in many cases allowing resolution of allelic combinations, which can be ambiguous when obtained using conventional approaches (Figure 7). Multiple pyrosequencing reactions, each performed with its own nucleotide dispensation order, frequently affect the resolution of allelic pairs (data not shown). Optimization of PSBT, through choice of dispensation order and selection of sequencing primer, improves genotype analysis. Selection of pyrosequencing primer is another critical variable for PSBT. Importantly, during pyrosequencing the choice of primers to promote specific initiation of DNA extension is sensitive to the choice of unique 3′-end, resulting from the lack of stringency associated with the optimal pyrosequencing reaction temperature. For alleles such as HLA-DRB, where more than 400 alleles have been reported and for HLA class I loci A and B exhibiting greater than 200 and 400 alleles, this is not a trivial problem. Computer algorithms have been used to scan for sets of small shared nucleotide sequences that are suitable for priming PSBT experiments, relying on selection of unique sequence motifs from which to match their 3′-end and lack of internal palindromic sequences.

Computer analysis of the HLA allelic sequences, obtained from the IMGT/HLA database [42], have been performed in order to identify suitable sequences for priming pyrosequencing. Additional group-specific primers will allow isolation of specific DRB signals from a population of amplified HLA genes, enabling preferential identification of those sequences during PSBT, avoiding the burden of multiple PCR preparations. Primers have been developed that are specific for a variety of DRB alleles including DRB1*03, *09 as well as DRB3 and DRB4 in a variety of genetic backgrounds (Figure 5). Data indicate that high resolution PSBT data

A

Allele	Motif Combination	Sequence Alignment
		Motif A *Motif B*
DRB1*0108	AxB	----------G-----A----G-A-----A----------A-----
		Motif C *Motif D*
DRB1*0406	CxD	----------C-----C----A-T-----C----------C-----
		Motif A *Motif D*
DRB1*010101	AxD	----------G-----A----G-A-----A----------C-----
		Motif C *Motif B*
DRB1*040101	CxB	----------C-----C----A-T-----C----------A-----

B

```
Dispensation  g_agcg__tgcg_t_c_gctg_a__cagatagcat_ctata_c_a_gc_a_g_ag_atc_acgtc_acgtgcgct_c
DRB1*0108     GGAGCGGGTGCGGTT--GCTGGAAA--GAT-GCAT-CTATAACCAAG--A-GGAG--T--ACGT----G--CGCTTC
DRB1*0406     GGAGCGGGTGCGGTTCC--TGGA--CAGATA-C-TTCTAT--C-A--CCAAG-AGGA-----GTCC--GTGCGCTTC
Signal        4_2226__2224_4_2_1124_4__1122211213_22222_3_3_12_3_3_23_110011222_002112224_2

DRB1*010101   GGAGCGGGTGCGGTT--GCTGGAAA--GAT-GCAT-CTATAACCAAG--A-GGAG--TCC--GT----G--CGCTTC
DRB1*040101   GGAGCGGGTGCGGTTCC--TGGA--CAGATA-C-TTCTAT--C-A--CCAAG-AGGA-----GT--ACGTGCGCTTC
Signal        4_2226__2224_4_2_1124_4__1122211213_22222_3_3_12_3_3_23_112_001100112112224_2
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FIGURE 7 Out-of-phase sequencing during PSBT can resolve cis-trans sequence elements during HLA genotyping. Panel A illustrates the HLA allele combinations DRB1*0108,*0406, and DRB1*010101,*040101 that traditional sequence-based typing is unable to resolve. However, during pyrosequencing these same alleles align to the dispensation order resulting in different PSBT sequencing signature (panel B). Abbreviations: HLA = human leukocyte antigen; PSBT = pyrosequence-based typing.

can be obtained using pyrosequencing primers that avoid internal palindromes and anneal to unique sites within the amplified PCR-derived template. Additional primer optimization occurred by combining a variety of approaches during design of PCR primers in which group specific amplification of DR4 was examined (Figure 5), including inhibition of pyrosequencing signal from co-amplified DRB pseudogene loci (Figure 5B) and the selection of forward PCR primer resulting in template DNA that avoided self-priming due to hybridization of its 3′-end with internal complementary sequence motifs [44]. Exploitation of these observations is anticipated to allow generation of PSBT data from HLA alleles improving the ability to identify the presence of a particular HLA genotype.

The ability to generate high resolution sequence data, and the throughput of hundreds of sequencing reactions per day, make PSBT an important method for identification of known genetic markers. Based on finding a short sequence in each allele of interest that occurs only once within the PCR generated fragment, the PSBT protocol allowed rapid identification of alleles for analysis of genetic predisposition to autoimmune disease as well as for histocompatibility typing. In studies of the genetic association of certain HLA alleles with the likelihood of developing autoimmune disease, as well as during clinical genotyping of patients and donors for histocompatibility matching during transplantation, PSBT is an ideal tool for rapid and accurate identification of HLA genotype.

ACKNOWLEDGMENTS

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Islet Cell Autoimmunity in a Triethnic Adult Population of the Third National Health and Nutrition Examination Survey

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Markers of humoral islet cell autoimmunity, such as autoantibodies (AAs) against the 65-kDa isoform of GAD (GAD65), serve as determinants of risk for autoimmune diabetes. Despite the high prevalence of diabetes in U.S. racial and ethnic minority adult populations, little is known concerning the prevalence of GAD65 AA in these groups. We estimated the prevalence of GAD65 AA in 1,064 diabetic and 1,036 nondiabetic participants who were 40–90 years of age from the Third National Health and Nutrition Examination Survey (NHANES III), which provides a representative ethnic sample of the U.S. diabetic population. The prevalence of GAD65 AA was higher in diabetic participants compared with nondiabetic participants in non-Hispanic whites (n = 920; 6.3% vs. 2.0%; P = 0.001) and non-Hispanic blacks (n = 534; 3.7% vs. 1.3%; P = 0.08) but not in Mexican Americans (n = 646; 1.2% vs. 2.6%; P = 0.18). Among diabetic non-Hispanic whites and non-Hispanic blacks, being GAD65 AA positive was associated with lower BMI and C-peptide (P < 0.05). These results may reflect the outcome of an autoimmune process leading to β-cell destruction/dysfunction in non-Hispanic white and non-Hispanic black adult diabetic patients as it occurs in a similar manner in type 1 diabetes. Among diabetic Mexican Americans, the lower prevalence of GAD65 AA suggests a lower frequency of autoimmune-related diabetes. ***Diabetes*** **53:1293–1302, 2004**

The prevalence of diabetes (diagnosed and undiagnosed) in adults ≥20 years of age in the U.S. was estimated to be 7.8% in the period between 1998 and 1994 based on data collected for the Third National Health and Nutrition Examination Survey (NHANES III) (1). It is projected that the prevalence of diabetes will continue to grow and that the largest increase will be among those aged ≥75 years (2,3). It has increasingly become more evident that in adults and the elderly, clinical criteria alone are no longer sufficient to permit an accurate distinction of type of diabetes (4,5). Markers of humoral islet cell autoimmunity seem to hold great promise as determinants of risk for autoimmune diabetes and for enhancing diabetes classification, even in older populations (6).

Antibodies to islet cell autoantigens, such as the 65-kDa isoform of GAD (GAD65), serve as predictors of disease onset and are found in 70–80% of children and adolescents before and at clinical diagnosis of type 1 diabetes (7,8). Autoantibodies (AAs) to GAD65, which serve as determinants of risk for autoimmune diabetes, seem to be present in up to 12% of adults who have a clinical diagnosis of type 2 diabetes (6,9–11). This form of diabetes with initial type 2 diabetes presentation but with evidence of islet cell autoimmunity has been termed latent autoimmune diabetes in adults, or type 1.5 diabetes, and has been associated with progressive decline in β-cell function and future insulin requirement in some populations (10,12–15).

Diabetes is more prevalent among non-Hispanic blacks and Mexican Americans than non-Hispanic whites (1). It is projected that the increase in diabetes will disproportionately affect non-Hispanic blacks and Hispanics (2,3). Despite the fact that in the U.S. the number of patients being diagnosed with type 2 diabetes is approaching an epidemic level, little is known concerning the prevalence of islet cell autoimmunity in these groups. The aim of the present study was to estimate the prevalence of GAD65 AAs in non-Hispanic white, non-Hispanic black, and Mexican-American adults who were previously evaluated as part of the NHANES III.

RESEARCH DESIGN AND METHODS

NHANES III. NHANES III was conducted by the National Center for Health Statistics (NCHS) of the Centers for Disease Control and Prevention from 1988 to 1994 (16). The survey included a nationally representative sample of the U.S. civilian noninstitutionalized population. A complex, stratified, multistage probability cluster sampling design was used with oversampling of blacks and Mexican Americans. The NHANES III survey consisted of a home interview followed by a physical examination in a mobile examination center (MEC). Details of this survey and methods of operation have been published (16).

During the home interview, data collected included medical history, health-related behavior, and sociodemographics. During this interview, individuals were asked whether they had a history of diabetes, their age at diagnosis, their use of diabetes medication, and their family history of diabetes. During the MEC examination, anthropometric assessments were performed. The MEC examinations could take place in the morning, after-

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AA, autoantibody; ADA, American Diabetes Association; GAD65, 65-kDa isoform of GAD; MEC, mobile examination center; NCHS, National Center for Health Statistics; NHANES, National Health and Nutrition Examination Survey; OHGA, oral hypoglycemic agent; WHO, World Health Organization.

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TABLE 1
Characteristics of study population by diabetic status and race/ethnicity, NHANES III participants (N = 2,100)*

	Non-Hispanic whites		Non-Hispanic blacks		Mexican Americans	
	No diabetes	Diabetes	No diabetes	Diabetes	No diabetes	Diabetes
n	491	429	239	295	306	340
Sex (% men)	260 (53)	220 (51)	118 (49)	118 (40)†	150 (49)	151 (44)
Age (years)	64 ± 12	69 ± 12‡	50 ± 11	62 ± 11†	57 ± 11	61 ± 11‡
BMI (kg/m^2)	27.7 ± 5.0	29.2 ± 5.7‡	28.6 ± 6.1	30.9 ± 6.5§	29.0 ± 4.8	29.7 ± 5.7
Fasting plasma glucose (mmol/l)	5.9 (5.4–6.3)	8.1 (6.4–11.4)‡	5.7 (5.2–6.3)	8.6 (6.4–13.0)‡	5.8 (5.4–6.3)	8.4 (6.2–13.4)‡
2-h glucose (mmol/l)	8.4 (6.5–9.7)	15.9 (11.6–20.2)‡	7.5 (5.8–9.3)	15.3 (10.6–20.5)‡	7.9 (6.1–9.5)	18.2 (13.3–22.8)‡
Fasting serum insulin (pmol/l)	64 (46–85)	106 (68–184)‡	62 (44–94)	118 (68–205)‡	70 (53–101)	95 (64–155)‡
HbA_{1c} (%)	5.4 (5.2–5.8)	6.9 (6.1–8.4)‡	5.7 (5.4–6.1)	7.5 (6.2–9.4)‡	5.6 (5.2–5.8)	7.7 (6.4–9.5)‡
C-peptide (pmol/ml)	0.86 (0.59–1.18)	1.19 (0.82–1.70)‡	0.76 (0.47–1.04)	0.92 (0.55–1.28)‡	0.92 (0.65–1.22)	1.11 (0.71–1.51)‡
Family history of diabetes (%)	204 (42.1)	253 (60.1)‡	91 (38.7)	193 (65.6)‡	129 (42.9)	219 (65.8)‡

*Data are n (%), mean ± SD, or median (interquartile range) for nonnormal variables. Diabetes status based on self-reported history of diabetes, diabetes medication use, or fasting ADA criteria. †$P < 0.05$, diabetic vs. nondiabetic participants. ‡$P < 0.0001$, diabetic vs. nondiabetic participants. §$P < 0.001$, diabetic vs. nondiabetic participants.

noon, or evening. For morning examinations, individuals 20 years of age or older were instructed to fast for 12 h. For the afternoon and evening examinations, individuals were instructed to fast for 6 h. Individuals who reported use of insulin were instructed not to fast. Fasting blood specimens obtained by venipuncture during the MEC examination were tested for lipid levels, glucose, insulin, C-peptide, and glycated hemoglobin concentration (HbA_{1c}). Plasma glucose and insulin levels 2 h after a 75-g (Dextol-75) oral glucose load were measured only in adult participants who were 40–74 years of age. A detailed description of laboratory techniques and quality assurance methods used in the NHANES III has been reported (16). NCHS placed a portion of the sera in a bank for unanticipated future research projects. With the permission of the NCHS, banked sera were used for this study.

Study population. All subjects included in the present study were part of the NHANES III. Available coded serum specimens (n = 2,182) from all eligible NHANES III participants with diabetes (n = 1,099) and a sample of nondiabetic participants (n = 1,083) ≥40 years of age were obtained from the NCHS and assayed for GAD65 AAs. The study population, for whom serum specimens were requested from the NCHS, was limited to NHANES III participants ≥40 years of age because the oral glucose tolerance test was performed only in adults who were 40–75 years of age and comparisons in the prevalence of GAD65 AA by both World Health Organization (WHO) and American Diabetes Association (ADA) diabetes diagnosis criteria were of interest (17,18). In this study population of NHANES III, individuals were classified with physician diagnosed diabetes if they reported a medical history of diabetes, including the utilization of insulin and oral hypoglycemic agents (OHGAs). Among participants not reporting a positive medical history of diabetes, individuals were diagnosed with diabetes using the ADA fasting glucose criteria (18). Based on these criteria, individuals with fasting glucose ≥126 mg/dl were classified as having undiagnosed diabetes. The prevalence of GAD65 AAs among new (undiagnosed) diabetic cases was similar irrespective of whether the ADA or WHO diabetes diagnosis criteria was used (data not shown). Therefore, all analyses in this article are presented in terms of the ADA diabetes diagnosis criteria.

Only serum samples from individuals with fasting ≥9 h were requested from NCHS and included in these analyses, therefore, participants attending the afternoon and evening sessions were not included in these analyses. A previous report by Harris et al. (1) did not find any statistically significant differences in sociodemographic and clinical variables between individuals assigned to the morning session as compared with those assigned to the afternoon/evening session. Moreover, for these analyses, 82 individuals of "other" race/ethnicity were excluded (47 nondiabetic and 35 diabetic participants).

The final NHANES III study population for this report consisted of 2,100 individuals, of whom 920 were non-Hispanic whites, 534 non-Hispanic blacks, and 646 Mexican Americans. The mean age was 63 years of age (SD = 12, range = 40–89) and the mean BMI was 29.1 (SD = 5.7). There were 1,017 men (48.4%) and 1,083 (51.6%) women in this NHANES III sample. Overall, non-Hispanic whites were leaner ($P < 0.0001$), older ($P < 0.001$), and had a greater percentage of males ($P < 0.05$) than non-Hispanic blacks and Mexican Americans.

Laboratory methods. AAs to GAD65 were detected in triplicate by immunobinding of serum with the in vitro transcribed/translated recombinant ^{35}S-[Met]-labeled recombinant human GAD65, as originally described by Grubin et al. (19). The GAD65 construct was kindly donated by Dr. Åke Lernmark. The results are expressed as an index (index = sample cpm − negative control cpm/positive control cpm − negative control cpm) as previously reported (6). The cutoff point for the assay was established as the 99th percentile of AA levels calculated using 280 control subjects for the radioimmunoassays and corresponded to 0.069. The cutoff point for being GAD65 AA positive used by our group is distinct from the cutoff point based on WHO standards and, as such, may not be directly comparable to other studies that use the WHO standard. The coefficient of variation of the GAD65 AA assay was previously reported (6). Results for our laboratory from proficiency workshops, organized by the University of Florida in Gainesville (1995–1997), the Diabetes Autoantibody Standardization Program (2000 and 2002), and WHO are summarized as follows: 76–100% sensitivity, 90–100% specificity (100% specificity three times), and 100% validity for GAD AAs (20).

Data analysis and statistical methods. Once the GAD65 AA assays were completed and results were returned to the NCHS, the sequence number associated with each serum sample, which then could be linked to the NHANES III database, was made available. All data presented in this article, with the exception of the GAD65 AA data, were originally obtained by NHANES III. The data were analyzed using Statistical Analysis System software (Release 8.00; SAS Institute, Cary, NC). Because of the complex race/ethnicity stratified sampling scheme used in NHANES III, most of the analyses are presented by race/ethnicity. Comparisons between groups were performed using the unpaired t test and ANOVA for normally distributed variables, the Mann-Whitney test for nonnormal variables, and the χ^2 test and Fisher's exact test, when appropriate, for categorical variables. Stepwise logistic regression analysis was used to assess independent correlates of GAD65 AA positivity within each racial/ethnic group. Variables included in the model were sex, age, BMI, diabetes status, fasting glucose and C-peptide levels, and HbA_{1c}. Models limited to diabetic groups also included diabetes duration and use of diabetes medication. All statistical tests were two tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Of the 1,064 diabetic individuals identified (n = 429 non-Hispanic whites, n = 295 non-Hispanic blacks, and n = 340 Mexican Americans), in 887 (83.4%) diabetes was physician diagnosed (reporting a positive history of diabetes). Of the 1,036 nondiabetic participants identified, 491 were non-Hispanic white, 239 were non-Hispanic black, and 306 were Mexican American. Characteristics of the study population by race/ethnicity and diabetes status are presented in Table 1. Diabetic participants across all racial/ethnic groups were older and had higher BMI and

TABLE 2
Characteristics of diabetic study population by race/ethnicity, NHANES III (n = 1,064)*

	Non-Hispanic whites	Non-Hispanic blacks	Mexican American	Overall P value
n	429	295	340	—
Sex (% men)	220 (51)†	118 (40)	151 (44)	0.009
Age (years)	69 ± 12†	62 ± 11	61 ± 11‡	<0.0001
BMI (kg/m^2)	29.2 ± 5.7†	30.9 ± 6.5§	29.7 ± 5.7	0.001
Fasting glucose (mmol/l)	8.1 (6.4–11.4)	8.6 (6.4–13.0)	8.4 (6.2–13.4)	NS
2-h glucose (mmol/l)‖	15.9 (11.6–20.2)	15.3 (10.6–20.5)§	18.2 (13.3–22.8)‡	0.002
Fasting insulin (pmol/l)¶	89 (62–133)	82 (55–133)	85 (56–126)	NS
HbA_{1c} (%)	6.9 (6.1–8.4)†	7.5 (6.2–9.4)	7.7 (6.4–9.5)‡	<0.0001
C-peptide (pmol/ml)	1.19 (0.82–1.70)†	0.92 (0.55–1.28)§	1.11 (0.71–1.51)‡	<0.0001
Diabetes by reported history (%)	344 (80.2)	245 (83.1)	298 (87.7)‡	0.02
Family history of diabetes (%)	253 (60.1)	193 (65.6)	219 (65.8)	NS
Physician-diagnosed diabetes#				
Age at diabetes diagnosis (years)	58 ± 13†	51 ± 13	51 ± 12‡	<0.0001
Diabetes duration (years)	11.1 ± 9.9	11.7 ± 10.7	10.6 ± 9.4	NS
Any diabetes medication (%)	258 (75.0)	191 (78.0)	223 (74.8)	NS
Insulin use (%)	103 (30)†	100 (41)§	70 (23)	0.0004
Use of OHGAs (%)	164 (48)	105 (43)§	167 (56)‡	0.02

*Data are n (%), mean ± SD, or median (interquartile range) for nonnormal variables. Diabetes status based on self-reported history of diabetes, diabetes medication use, or fasting ADA criteria. †$P < 0.05$, non-Hispanic white vs. non-Hispanic black. ‡$P < 0.05$, non-Hispanic white vs. Mexican American. §$P < 0.05$, non-Hispanic black vs. Mexican American. ‖2-h glucose data are based on 176 non-Hispanic whites, 141 non-Hispanic blacks, 201 Mexican Americans. ¶Excludes insulin users, resulting in insulin data on 323 non-Hispanic whites, 195 non-Hispanic blacks, 268 Mexican Americans. #Following data are based on a group of individuals with positive history of diabetes only (344 non-Hispanic whites, 245 non-Hispanic blacks, 298 Mexican Americans).

higher levels of fasting glucose and insulin (consistent results were obtained when excluding insulin users), 2-h glucose, HbA_{1c}, and C-peptide, and a greater percentage reported a positive family history of diabetes as compared with nondiabetic participants.

Table 2 presents demographic and clinical characteristics among participants with and without physician-diagnosed diabetes by race/ethnicity. Among diabetic participants, a greater percentage of non-Hispanic whites were male as compared with non-Hispanic blacks. Compared with non-Hispanic white diabetic participants, Mexican-American and non-Hispanic black diabetic participants were younger at the time of the survey and at diagnosis of diabetes and had higher levels of HbA_{1c} ($P < 0.05$). Moreover, non-Hispanic blacks were heavier than non-Hispanic whites and Mexican Americans. A greater percentage of Mexican Americans reported a history of diabetes (88%) as compared with non-Hispanic blacks (83%; NS) and non-Hispanic whites (80%; $P < 0.05$). Among participants with diagnosed diabetes, there was no statistically significant difference by race/ethnicity in duration of diabetes or the percentage of participants who reported use of any diabetes medication. However, Mexican-American patients (56%) were more frequently treated with OHGAs compared with non-Hispanic whites (48%; $P < 0.05$) and non-Hispanic blacks (43%; $P < 0.05$) and less frequently with insulin compared with non-Hispanic blacks (41%; $P < 0.05$).

Sixty-three individuals were classified as GAD65 AA positive in our study population (37 non-Hispanic whites, 14 non-Hispanic blacks, and 12 Mexican Americans). The overall prevalence of GAD65 AA positivity among diabetic (physician-diagnosed and undiagnosed diabetes) individuals (4.0%; $n = 42$) was statistically significantly ($P = 0.01$) higher than that in nondiabetic individuals (2.0%, $n = 21$). The prevalence of GAD65 AAs was higher in the non-Hispanic white (6.3% vs. 2.0%; $P = 0.001$; diabetes versus no diabetes) and non-Hispanic black (3.7% vs. 1.3%; $P = 0.08$) diabetic population compared with the nondiabetic population, a difference not evident in Mexican Americans (1.2% vs. 2.6%; $P = 0.18$; Fig. 1). This racial/ethnic pattern in the prevalence of GAD65 AAs was also seen when the analysis was limited to participants with physician-diagnosed diabetes (Fig. 1). The difference in prevalence of GAD65 AAs by diabetes status was found in both men and women, but the difference in GAD65 AA positivity by diabetes status was more pronounced in non-Hispanic white women and only statistically significant in this group (Fig. 2).

We further compared the prevalence of GAD65 AA positivity by diabetes status in different age-groups. In non-Hispanic whites, the prevalence of GAD65 AAs was consistently higher in diabetic versus nondiabetic individuals, with the exception of the oldest group (≥75 years of age; Fig. 3 and Table 3). It is interesting that in non-Hispanic blacks, the difference in GAD65 AA positivity by diabetic status was evident only in the older age-groups (>60 years of age; Fig. 3).

We then divided participants with physician-diagnosed diabetes ($n = 870$) into four groups by duration of diabetes as follows: 0–3 years ($n = 234$, 26.9%), 4–9 years ($n = 216$, 24.8%), 10–16 years ($n = 203$, 23.3%), and ≥17 years ($n = 217$, 24.9%). Among non-Hispanic whites, GAD65 AA prevalence rates were 8.6, 5.0, 5.0, and 8.0% in the group with 0–3, 4–9, 10–16, and ≥17 years' duration of diabetes, respectively. Among non-Hispanic blacks, GAD65 AA prevalence rates were 4.5, 3.9, 1.8, and 7.9% in the group with 0–3, 4–9, 10–16, and ≥17 years' duration of diabetes, respectively. In Mexican Americans, GAD65 AA positivity was limited to the first two groups of duration of diabetes, 0–3 years (2.7%) and 4–9 years (1.2%), with none of the diabetic participants in the groups of 10–16 and ≥17 years'

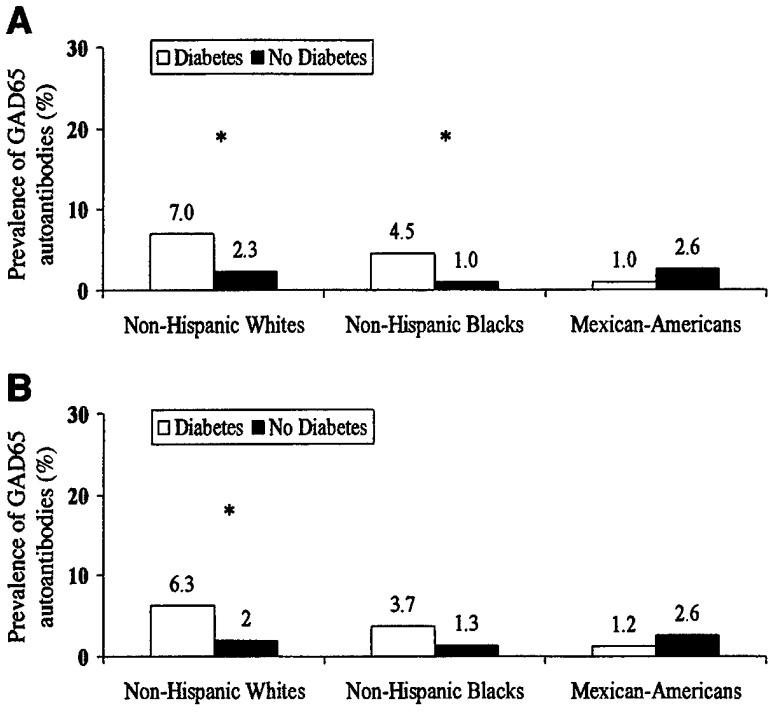


FIG. 1. Prevalence (%) of GAD65 AAs by diabetes status and race/ethnicity among adults ≥40 years of age, NHANES III population (N = 2,100), *P < 0.05. *A*: Physician-diagnosed diabetes. *B*: Physician-diagnosed and undiagnosed diabetes.

duration of diabetes positive for GAD65 AAs. None of these race-specific comparisons of GAD65 AA positivity by duration of diabetes were statistically significant. Similar results were obtained when including both participants with and without physician-diagnosed diabetes (data not shown).

Although the prevalence of GAD65 AAs was higher in non-Hispanic white and non-Hispanic black diabetic participants as compared with their nondiabetic counterparts, differences were attenuated for certain subgroups (as shown in Table 3). Non-Hispanic white diabetic participants were more likely to be GAD65 AA positive compared with nondiabetic participants when analyses were limited to participants <75 years of age and even when the diabetic group was limited to the following subgroups: *1*) those who diabetes was diagnosed after age 40, *2*) diabetic participants who were not on insulin, and *3*) those who had C-peptide levels ≥0.2 pmol/ml (as a cutoff point for insulin deficiency) and were not on insulin (21). Differences in GAD65 AA positivity by diabetes status persisted in non-Hispanic whites and non-Hispanic blacks when analyses were limited to *1*) participants with C-peptide

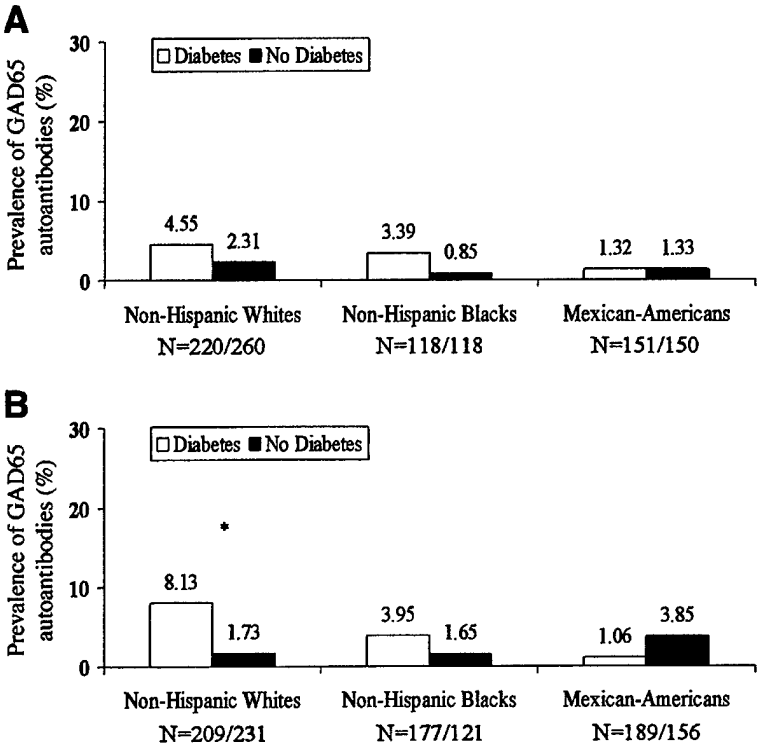


FIG. 2. Prevalence (%) of GAD65 AAs by self-reported diabetes status, fasting ADA criteria, and race/ethnicity among male and female adults ≥40 years of age, NHANES III population (N = 2,100), *P < 0.05. *A*: Men (n = 1,017). *B*: Women (n = 1,083).

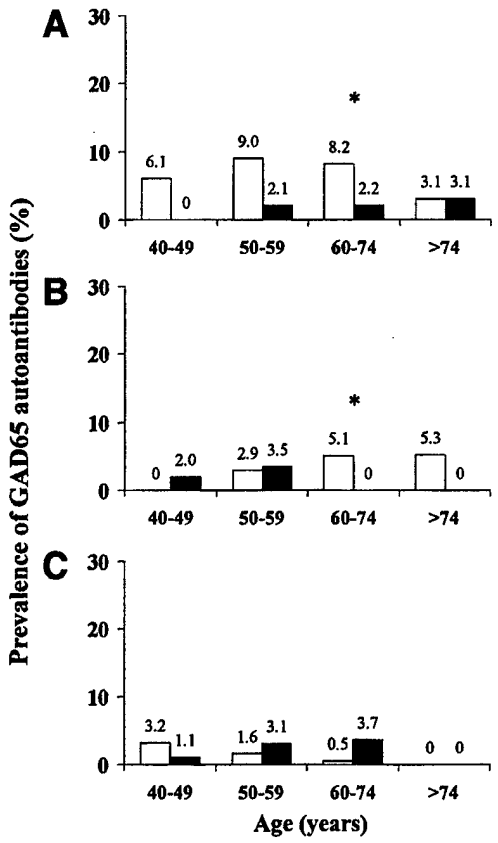


FIG. 3. Prevalence of GAD65 AAs by self-reported diabetes status and fasting ADA criteria, age and race/ethnicity among adults ≥40 years of age, NHANES III population (N = 2,100), *P < 0.05. *A*: Non-Hispanic whites (n = 920). *B*: Non-Hispanic blacks (n = 534). *C*: Mexican Americans (n = 646). □, diabetes; ■, no diabetes.

levels ≥0.2 pmol/ml (not statistically significant in non-Hispanic whites) and *2*) diabetic participants who were on insulin. There is consistently no difference in the prevalence of GAD65 AAs by diabetic status for Mexican Americans.

We also looked at the prevalence of GAD65 AAs by time from diagnosis of diabetes to initiation of insulin therapy. For these analyses, data on current duration of insulin use were used to estimate time from diagnosis to initiation of insulin therapy among current insulin users. Data on current duration of insulin use was available for 265 (97.8%) of the 271 current insulin users. Among current insulin users, the prevalence of GAD65 AAs was 7.5, 5.4, 8.0, and 6.8% in individuals who initiated insulin therapy 0, 1–2.9, 3–9.9, and ≥10 years after diagnosis of diabetes (P = 0.97). GAD65 AA prevalence data by race/ethnicity were limited by the small number of insulin users in each racial/ethnic category.

We then compared GAD65 AA–positive diabetic individuals with GAD65 AA–negative diabetic individuals and found that GAD65 AA positivity was associated with lower BMI, higher HbA_{1c}, and lower C-peptide among non-Hispanic whites and non-Hispanic blacks (Table 4). Non-Hispanic black diabetic participants who were GAD65 AA positive were more likely to have higher fasting glucose levels. Among non-Hispanic whites, the percentage of diabetic patients who were treated with insulin was higher in GAD65 AA–positive as compared with GAD65 AA–negative patients (P < 0.05). A similar pattern was also present in non-Hispanic blacks, although the difference was not statistically significant (Table 4). There were no statistically significant differences with regard to clinical characteristics between GAD65 AA–positive and –negative diabetic Mexican Americans, although GAD65 AA–positive diabetic Mexican Americans seem to have lower insulin and C-peptide levels. Moreover, although there were no statistically significant differences in duration of diabetes by GAD65 AA status in any of the racial/ethnic groups, GAD65 AA–positive diabetic non-Hispanic blacks tended to have longer duration of diabetes compared with their GAD65 AA–negative counterparts. Among diabetic participants, the prevalence of GAD65 AA did not differ statistically by duration of insulin treatment. The prevalence of GAD65 AAs was 4.9, 6.7, 4.3, and 6.8% among participants with prevalent diabetes with 0–2, 2–5, 5–10, and >10 years of insulin use, respectively.

We then evaluated the relationship between GAD65 AAs and some markers of insulin secretion. The mean (±SD)

TABLE 3
Prevalence (%) of GAD65 AA positivity by diabetes status and race/ethnicity among subgroups, NHANES III participants

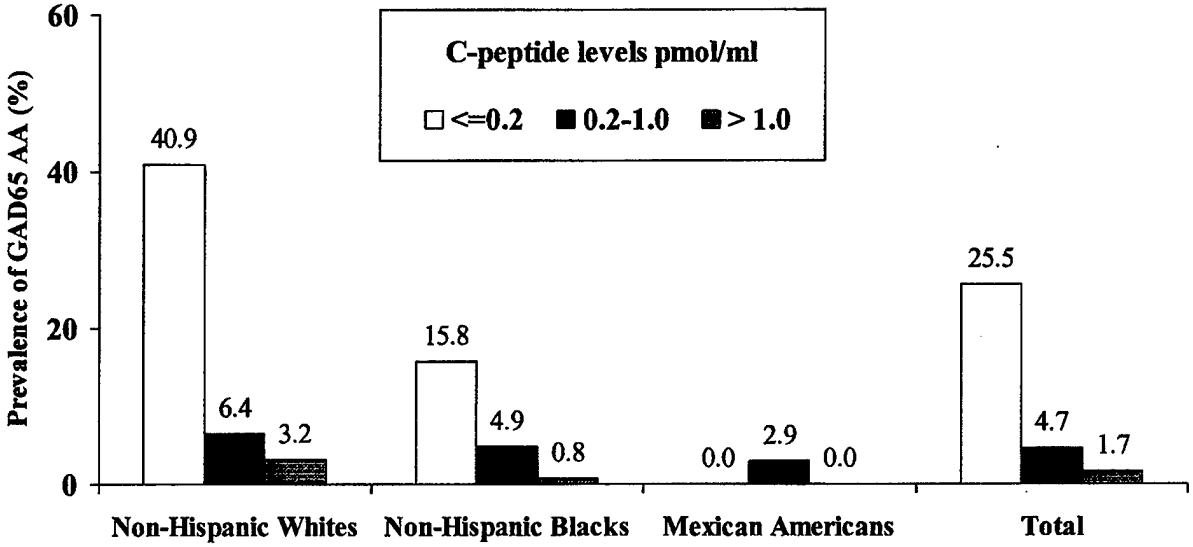
	Non-Hispanic whites		Non-Hispanic blacks		Mexican Americans	
	No diabetes	Diabetes	No diabetes	Diabetes	No diabetes	Diabetes
All diabetes*						
n	491	429	239	295	306	340
Participants age <75 years	1.8	8.2†	1.4	3.5	2.7	1.3
Participants age ≥75 years	3.1	3.1	0	5.3	0	0
C-peptide ≥ 0.2 pmol/ml	2.3	4.4	0.7	3.5†	2.6	1.0
Physician-diagnosed diabetes at ≥40 years of age‡						
n	491	402	239	248	306	296
All participants	2.0	5.7†	1.3	3.2	2.6	1.4
Diabetic participants not on insulin	2.0	4.8†	1.3	2.3	2.6	1.3
Diabetic participants on insulin	2.3	8.7†	1.0	5.4†	2.6	1.7
Participants with C-peptide ≥0.2 pmol/ml	2.1	4.4†	0.9	3.0	2.6	1.4
Diabetic participants not on insulin and C-peptide ≥0.2 pmol/ml	2.1	4.6†	0.9	2.4	2.6	1.3

*Diabetes status based on self-reported history of diabetes, diabetes medication use, or fasting ADA criteria. †P < 0.05, diabetic group vs. nondiabetic group. ‡Diabetes status based on positive history of diabetes only (self-reported history of diabetes and current diabetes treatment), and participants in diabetes group limited to those diagnosed after the age of 40.

TABLE 4
Characteristics of diabetic participants by race/ethnicity and GAD AA positivity, NHANES III participants (n = 1,064)*

	Non-Hispanic whites		Non-Hispanic blacks		Mexican Americans	
	GAD65 AA^+	GAD65 AA^-	GAD65 AA^+	GAD65 AA^-	GAD65 AA^+	GAD65 AA^-
n	27	402	11	284	4	336
Sex (% men)	37	52.2	36.4	40.1	50	44.4
Age (years)	65 ± 11	69 ± 12	68 ± 8	62 ± 12	53 ± 8	61 ± 11
BMI (kg/m^2)	27.2 ± 5.9	29.4 ± 5.7†	25.1 ± 4.5	31.1 ± 6.5†	28.1 ± 1.9	29.7 ± 5.7
Fasting glucose (mmol/l)	8.2 (6.6–14.5)	8.0 (6.4–11.4)	15.6 (8.5–19.4)	8.4 (6.4–12.9)†	8.2 (6.5–12.3)	8.4 (6.2–13.4)
2-h glucose (mmol/l)	16.4 (15.4–21.7)	15.8 (11.4–20.2)	18.9 (6.2–31.5)	15.2 (10.5–20.5)	18.4 (9.8–22.8)	18.2 (13.3–22.9)
Fasting insulin (pmol/l)‡	21.9 (9.8–47.6)	17.6 (11.4–30.5)	24.6 (8.0–62.0)	19.7 (11.3–33.2)	10.8 (7.7–19.4)	16.0 (10.8–25.9)
HbA_{1c} (%)	8.3 (6.2–9.1)	6.9 (6.0–8.3)†	9.4 (7.6–10.7)	7.5 (6.2–9.4)†	7.7 (5.5–10.5)	7.7 (6.4–9.5)
C-peptide (pmol/ml)	0.74 (0.11–1.15)	1.21 (0.84–1.71)†	0.47 (0.02–0.78)	0.92 (0.60–1.29)†	0.73 (0.63–0.80)	1.12 (0.72–1.52)
Systolic blood pressure (mmHg)	134 ± 20	141 ± 19	141 ± 19	141 ± 19	134 ± 23	141 ± 23
Diastolic blood pressure (mmHg)	73 ± 12	73 ± 10	76 ± 8	77 ± 11	81 ± 7	75 ± 11
LDL cholesterol (mmol/l)	2.77 (2.33–3.31)	3.59 (2.97–4.22)†	3.41 (3.13–3.70)	3.59 (3.03–4.37)	3.15 (2.82–3.47)	3.41 (2.84–4.01)
HDL cholesterol (mmol/l)	1.24 (0.83–1.66)	1.06 (0.88–1.32)	1.11 (0.85–1.81)	1.24 (1.03–1.55)	1.09 (1.01–1.19)	1.16 (0.98–1.37)
Triglycerides (mmol/l)	3.85 (2.53–7.71)	4.86 (3.44–7.37)	3.00 (1.86–4.58)	3.75 (2.48–5.25)	4.71 (2.66–5.35)	4.60 (3.44–7.06)
Physician-diagnosed diabetes (n = 887)§						
n	24	320	11	234	3	295
Age at diabetes diagnosis (years)	55 ± 13	58 ± 13	52 ± 16	51 ± 13	51 ± 8	51 ± 12
Diabetes duration (years)	11.1 ± 10.5	11.1 ± 9.8	16.2 ± 11.5	11.5 ± 10.5	3.7 ± 2.1	10.7 ± 9.4
Use of any diabetes medication (%)	79.2	74.7	90.9	77.4	66.7	74.9
Insulin use (%)	50	28.4†	63.6	39.7	33.3	23.4
OHGA use (%)	29.2	49.1†	54.6	42.3	33.3	56.3

*Data are percentage, mean ± SD, or median (interquartile range) for nonnormal variables. Diabetes status based on self-reported history of diabetes, diabetes medication use, or fasting ADA criteria. †$P < 0.05$, GAD65 AA^+ vs. GAD65 AA^-. ‡Excludes insulin users, resulting in insulin data on 15 GAD65 AA^+ and 310 GAD65 AA^- non-Hispanic whites, 4 GAD65 AA^+ and 191 GAD65 AA^- non-Hispanic blacks, 3 GAD65 AA^+ and 267 GAD65 AA^- Mexican Americans. §Diabetes status based on positive history of diabetes only (self-reported history of diabetes and current diabetes treatment).



GAD65AA +	9	8	9	3	7	1	0	4	0	12	19	10
N	22	125	279	19	144	132	6	139	193	47	408	604
P-value *	< 0.001			0.006			0.07			<0.0001		

FIG. 4. Prevalence of GAD65 AAs by C-peptide levels (pmol/ml) and race/ethnicity among all participants with diabetes, NHANES III (n = 1,059). *Overall race/ethnicity-specific comparisons

C-peptide levels was 1.05 ± 0.61 pmol/ml for the total population (n = 2,092; C-peptide levels were missing for 9 individuals). Seventy-three participants had C-peptide level ≤0.2 pmol/ml, 47 of whom had a history of diabetes and 26 of whom had no history of diabetes and were considered nondiabetic on the basis of ADA fasting criteria. Of the 47 with diabetes, 36 were insulin users, 5 used both insulin and OHGAs, and 2 used OHGAs only. Fourteen individuals had C-peptide levels equal to 0.021 pmol/ml (minimum detection limit), 11 of whom reported a history of diabetes (all insulin users) and 3 of whom had no history of diabetes and had fasting glucose <126 mg/dl. Among non-Hispanic white and non-Hispanic black diabetic participants, the prevalence of GAD65 AAs was significantly higher with decreasing levels of C-peptide (Fig. 4). Moreover, among the 21 nondiabetic individuals who were GAD65 AA positive, GAD65 AA titers were correlated with 2-h glucose (r_s = 0.49, P = 0.04, n = 18) and fasting insulin (r_s = 0.41, P = 0.07, n = 21). Among the 21 GAD65 AA–positive nondiabetic participants, 2 (9.5%) had 2-h glucose levels >200 mg/dl and 6 (28.9%) had fasting glucose levels >110 mg/dl. In addition, 10 (48%) had a family history of diabetes.

Stepwise logistic regression modeling of the total population resulted in the following variables being significantly independently associated with GAD65 AA positivity: lower C-peptide levels, white race, and lower BMI. This was a consistent finding regardless of diabetes status variable used and regardless of whether the analysis was limited to the diabetic or total population. In non-Hispanic whites, diabetes status and low C-peptide levels were significantly independently associated with GAD65 AA positivity. In non-Hispanic blacks, low C-peptide levels and low BMI were independently associated with GAD65 AA positivity. Duration of diabetes and use of diabetes medication were not independently associated with GAD65 AA positivity among the diabetic population. No variables were statistically significantly associated with GAD65 AA positivity in Mexican Americans.

DISCUSSION

Type 2 diabetes is the most common form of diabetes, accounting for ~90% of cases and in many industrialized countries affecting 10–20% of individuals aged >45 years (22). The number of patients being diagnosed with type 2 diabetes is increasing each year and is approaching an epidemic level; in adults, the prevalence of diabetes worldwide was estimated to be 4.0% in 1995 and it is projected to rise to 5.4% by 2025 (23). Type 2 diabetes is a heterogeneous disorder of glucose and metabolic homeostasis that is characterized by an intricate interaction between insulin resistance and pancreatic β-cell dysfunction (24).

In a subgroup of type 2 diabetic patients, there are signs of humoral islet cell autoimmunity (25–27). To date, GAD65 AAs represent the most commonly detected marker in this subgroup of type 2 diabetic patients (28–30) and seem to be present in up to 12% of adults who received an initial diagnosis of type 2 diabetes (6,9,10,31). On the basis of these and older studies, it has been proposed that this subgroup of type 2 diabetes with islet cell autoimmunity may represent a different disease with type 1–like autoimmune pathogenesis. This form of diabetes with initial type 2 diabetes presentation is termed latent auto-

immune diabetes in adults, type 1.5 diabetes, or slowly progressive type 1 diabetes and has been associated with progressive decline in β-cell function and future insulin requirement (10,12–15). This is significant when one considers the extraordinarily high prevalence of diabetes in U.S. adults and that up to 12% of these adults may actually have a diabetes of autoimmune nature.

Despite the high prevalence of type 2 diabetes in U.S. racial/ethnic minority adult populations, little is known concerning the prevalence of GAD65 AAs in these groups. We measured one of the most widely used markers for the diagnosis and prediction of type 1 diabetes, GAD65 AA (7,32,33), in a well-characterized diabetic and nondiabetic population aged ≥40 years from the NHANES III. We observed that the prevalence of GAD65 AAs was higher in diabetic participants compared with nondiabetic participants in non-Hispanic whites and non-Hispanic blacks but not in Mexican Americans. The prevalence of GAD65 AAs in the triethnic adult population of NHANES III ranged from 1.2% in diabetic Mexican Americans to 6.3% in diabetic non-Hispanic whites. On the basis of our GAD65 AA prevalence estimates, data on the prevalence of diabetes in the U.S. reported by NHANES III, and the projected U.S. population for 2002 (34), we estimated that 720,000 non-Hispanic white, 67,000 non-Hispanic black, and 13,000 Mexican-American adults 40–74 years of age in the U.S. have evidence of islet cell autoimmunity, a prevalence as great as that of type 1 diabetes.

The prevalence of GAD65 AAs in different adult populations seems to be variable, being higher in Northern Europeans (10,25,31) and lower in Alaskan natives (35), Pima Indians (36), and populations from Northern Italy (37) and Southern Spain (38). It is not entirely clear why there is such a variation. Both genetic background and sample size of the populations examined in each study may account for these differences. In addition, the age of type 2 diabetic patients evaluated for GAD65 AAs or the inconsistency between studies about the criteria applied to exclude patients with clinical features of type 1 diabetes may explain differences in the prevalence reported in these studies. In our study, the prevalence of GAD65 AAs among diabetic whites was somewhat lower than that reported by other studies of white populations, including our study of older diabetic participants of the Cardiovascular Health Study (6,10,31). Similarly, the prevalence of GAD65 AAs in diabetic non-Hispanic blacks from the NHANES III was lower than that reported for blacks from the Cardiovascular Health Study (6).

We believe that one reason why Mexican Americans who are ≥40 years of age have lower prevalence of autoimmune phenomena compared with other American populations of white or black descent is because the incidence of type 1 diabetes in Mexican Americans is very low (39). In support of these findings, Hathout et al. (40) evaluated the prevalence of islet cell antibodies and GAD and insulin AAs at clinical diagnosis of type 2 diabetes in a group of white and Hispanic children and adolescents. They found that none of the Hispanic children with type 2 diabetes exhibited signs of humoral islet cell autoimmunity. A distinct genetic background in Mexican Americans compared with whites and blacks and differences in environmental factors such as diet and visceral adiposity, which are major determinants of the development of type 2 diabetes in Mexican Americans, are possible reasons for the difference in the prevalence of GAD65 AAs (41,42).

The possibility that other antibodies to islet autoantigens (which were not measured in this study) may be present in Mexican Americans cannot be excluded. We are currently measuring AAs to another islet cell antigen, insulinoma-associated protein 2 (43), in the NHANES III population. However, as we and others have shown, insulinoma-associated protein 2 AAs alone are not a strong marker for autoimmune diabetes in older-onset diabetes cases and tends to decrease with age to a much greater extent than GAD65 AAs, which tend to be much more stable in adults with diabetes (6,9). Given the cross-sectional nature of this study and that both new and prevalent diabetic individuals were identified, we believed that GAD65 AAs would be the first antibody marker of choice to detect in this population. Although data on sequential determination of GAD65 AAs are not available for this study, there is strong evidence to suggest that GAD AAs are stable for a number of years or even decades in patients who present with both type 1 (44,45) and type 2 diabetes (31,46).

Several key findings from our evaluation of the NHANES III population support the role of the outcome of an autoimmune process leading to β-cell damage and subsequent reduced insulin secretion in non-Hispanic whites and non-Hispanic blacks as it occurs in a similar manner in type 1 diabetes. Among diabetic non-Hispanic whites and non-Hispanic blacks, there was evidence of islet cell autoimmunity as measured through the presence of GAD65 AAs. We also found that among non-Hispanic whites and non-Hispanic blacks, the prevalence of GAD65 AAs was dramatically higher in diabetic individuals with low fasting levels of C-peptide, indicative of insulin secretion dysfunction. We also found that in non-Hispanic whites and non-Hispanic blacks, GAD65 AA positivity was independently associated with lower BMI and C-peptide levels, findings that are consistent with other studies (9,11).

In conclusion, in patients who are diagnosed with adult-onset diabetes, clinical and metabolic parameters alone may no longer be considered sufficient to allow an adequate classification of diabetes (27). The classification of diabetes continues to undergo changes, and markers of autoimmunity may be another tool for improving diabetes classification, which is important given the dependence of diabetes treatment on classification and initial diagnosis of diabetes. Furthermore, a more appropriate classification of diabetes will shed light on the heterogeneous pathoetiologic factors associated with autoimmune diabetes.

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SOP^3: a web-based tool for selection of oligonucleotide primers for single nucleotide polymorphism analysis by Pyrosequencing®

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SOP^3 is a web-based software tool for designing oligonucleotide primers for use in the analysis of single nucleotide polymorphisms (SNPs). Accessible via the Internet, the application is optimized for developing the PCR and sequencing primers that are necessary for Pyrosequencing®. The application accepts as input gene name, SNP reference sequence number, or chromosomal nucleotide location. Output can be parsed by gene name, SNP reference number, heterozygosity value, location, chromosome, or function. The location of an individual polymorphism, such as an intron, exon, or 5′ or 3′ untranslated region is indicated, as are whether nucleotide changes in an exon are associated with a change in an amino acid sequence. SOP^3 presents for each entry a set of forward and biotinylated reverse PCR primers as well as a sequencing primer for use during the analysis of SNPs by Pyrosequencing. Theoretical pyrograms for each allele are calculated and presented graphically. The method has been tested in the development of Pyrosequencing assays for determining SNPs and for deletion/insertion polymorphisms in the human genome. Of the SOP^3-designed primer sets that were tested, a large majority of the primer sets have successfully produced PCR products and Pyrosequencing data.

INTRODUCTION

Pyrosequencing® has been developed to allow for accurate sequencing of short stretches of DNA, initially for the analysis of expressed sequence tags (ESTs; References 1–3). Sequencing of lengths between 50 and 150 nucleotides has been reported during HLA genotyping as well as during the analysis of PCR-amplified cloned DNA (4–7). The method is suited for use during the analysis of single nucleotide polymorphisms (SNPs) and the detection of genomic insertion and deletion polymorphisms because of its ability to provide high-quality sequencing for short stretches of DNA and to accurately resolve heterozygous nucleotides by enabling out-of-phase sequencing (8). Pyrosequencing is performed by the addition of dNTPs individually, in a predefined dispensation order, so that the nascent nucleotide chain is extended one nucleotide residue per dispensation event. The detection of nucleotide sequence is performed by way of a chain of enzymatic reactions involving the activities of DNA polymerase, apyrase, ATP sulfurylase, as well as luciferase (1,5). The incorporation of a particular nucleotide is displayed graphically in the form of a pyrogram of nucleotide dispensation event versus the intensity of emitted light. The Pyrosequencing reaction is quantitative in that increased light intensity is produced upon incorporation of multiple nucleotides.

The design of oligonucleotide primer sets for use during PCR and Pyrosequencing, a necessary step in the process of Pyrosequence-based mapping of genetic disease loci, while not individually cumbersome, is time-consuming to develop and test when large numbers of SNP-containing sites are to be examined. Publicly and commercially available software applications have been developed for PCR primer design, but these have not taken into account the added constraints that are essential when optimizing Pyrosequencing reagents (9,10). Moreover, applications currently available for developing Pyrosequencing primers do not also allow for the design of primers for PCR. Thus, none of the existing software applications available via the Internet are capable of designing the complete set of primers required for Pyrosequence-based typing (PSBT) during SNP-scanning projects while also allowing for the simultaneous development of assays for multiple SNPs. The development of Pyrosequencing assays can be accomplished by exploitation of the sequence databases derived from the human and model organism genome projects and can be combined with primer design algorithms when large batches of SNPs are to be analyzed. Assay development relies on the availability of genome databases containing the identity and location of relevant SNPs for input into primer design software applications. Currently, this can be achieved only by

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transporting the data between several software applications, each of which is specialized for use at a different stage of the process. Combining these steps into a single software application can improve the efficiency of the experimental design, increasing the level of data integrity by avoiding the use of different software applications to accomplish each of these goals independently (11).

This paper presents software that can efficiently design PCR and Pyrosequencing primers for large numbers of SNPs. The method automates existing processes and takes into consideration restrictions that should be applied during Pyrosequencing (1,2,5–7,12). Moreover, the application aids in the sorting of SNPs for biologically interesting properties (e.g., the association of an SNP with a particular genetic structural element, whether it codes for an amino acid change in the resulting protein, and its heterozygosity within a population), which, in turn, can add to the usefulness of particular primer sets during physical mapping studies.

MATERIALS AND METHODS

Selection of Oligonucleotide Primers for PCR and Pyrosequencing (SOP^3)

The SOP^3 application consists of warehoused genomic sequence data from the human genome project downloaded from the UCSC Genome Browser and the National Center for Biotechnology Information (NCBI) human genome release, Build 34, of the finished human genome assembly (http://hgdownload.cse.ucsc.edu/glodenPath/hg16/bigZips). Written in preprocessor hypertext protocol (PHP; Zend Technologies Ltd., Ramat Gan, Israel), the SOP^3 application and associated MySQL database were developed on a Linux SUSE Enterprise Server 8 for the AMD64 operating system with Apache version 2.0.48 (Apache Software Foundation, Forest Hill, MD, USA) on a customized computational computer server (@Xi Computer, San Clemente, CA, USA) consisting of 2× AMD Opteron™ 246 64-Bit processors with 1024 kb Cache, 8192 MB random access memory (RAM), and equipped with 3× 250 GB drives. The application and associated warehoused databases were designed to run as an Internet-available web site.

Preparation of Genomic DNA

Samples of genomic DNA were obtained either from purchased human genomic DNA (Human Biological Data Interchange, Philadelphia, PA, USA) or whole blood extractions from healthy donor volunteers. Purification of genomic DNA was performed using a QIAamp® DNA Mini Kit (Qiagen, Valencia, CA, USA) as directed by the manufacturer. DNA yields were typically greater than 5 µg of genomic DNA per sample. The samples were stored frozen at -20°C and thawed immediately prior to use.

PCR

Oligonucleotides and 5′-end biotinylated oligonucleotides, examples of which are listed in Supplementary Table S1 available at the *BioTechniques*' web site at http://www.BioTechniques.com/December2004/AlexanderSupplementary.html, were purchased from Integrated DNA Technologies (Coralville, IA, USA). PCR amplification (13) was performed in 50 µL volumes containing *Taq* buffer, 2 mM $MgCl_2$, 0.2 µM each dNTP (buffers and nucleotides were purchased from Applied Biosystems, Foster City, CA, USA), 0.2 µM forward and biotinylated reverse primers, 1 U *Taq* polymerase, and 5 µL purified genomic DNA (roughly 10 ng DNA). Amplification included 96°C incubation for 3 min, followed by 50 cycles at 96°C, 55°C, and 72°C, incubated for 30 s at each step. PCR cycling was followed by a final 5-min incubation at 72°C. The samples were then stored at -20°C or 4°C prior to Pyrosequencing. Amplification was performed using the primer sets designed by the customized software application SOP^3, a subset of which are listed in supplementary Table S1.

Pyrosequencing

The Pyrosequencing aparatus was purchased from Pyrosequencing AB (Uppsala, Sweden). Pyrosequencing reactions were performed using reagents provided with the PSQ™ 96 Sample Preparation kit and the PSQ 96 SQA Reagent kit (Pyrosequencing AB). Briefly, samples of 20–40 µL amplified DNA from the PCR mixture were mixed with 4 µL streptavidin-coated beads (Amersham Biosciences, Piscataway, NJ, USA) and prepared for Pyrosequencing as recommended by the manufacturer. The appropriate Pyrosequencing primer was added to each well in a volume of 5 µL using a 3-µM stock solution. The samples were heated to 80°C for 2 min, and then allowed to cool for 5 min at room temperature and sequenced by Pyrosequencing. A detailed description of the Pyrosequencing reaction conditions has been reported by Gharizadeh et al. (5). Pyrosequencing data were quantified and background corrected using PSQ 96MA version 2.0.2 software (Pyrosequencing AB).

RESULTS

Algorithm for the Selection of PCR Primers

Designed to accept input via a web-based user interface, the software application SOP^3 can be accessed over the Internet at URL http://biodev.hgen.pitt.edu/sop3/. The application consists of a database of the human genome sequence downloaded from the UCSC Genome Browser, Build 34, integrated with a human SNP database obtained from dbSNP, Build 118, at NCBI. The software application is written in PHP and enables user-defined queries to be examined against the SNP and sequence databases. As illustrated in the flowchart (Figure 1), the application accepts input as a gene name, SNP reference sequence number, or chromosomal nucleotide location. Multiple gene names or reference sequences can be queried simultaneously with the upper limit currently set at 1000 SNPs. When a query is presented, the application extracts that information and compares it to the warehoused database. FASTA-formatted sequences of user-selectable length flanking the SNP (maximum of 2000 nucleotides), along with its associated attributes (e.g., SNP location within a genetic structural element and hetero-

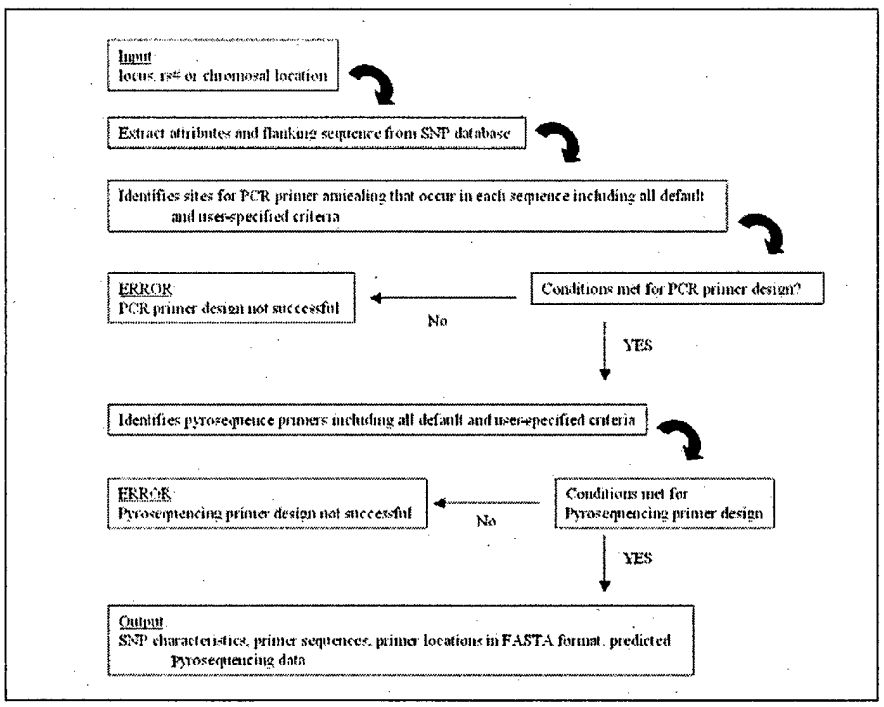


Figure 1. Representation of the organization of the SOP[3] application web site. Data were combined from the UCSC Genome Browser (Build 34) and dbSNP (Build 118) from the National Center for Biotechnology Information (NCBI) into the SOP[3] database. The SOP[3] application enables the analysis of SNP attributes, including flanking sequence and chromosomal location, during the design of primer sets for PCR and Pyrosequencing. SNP, single nucleotide polymorphism.

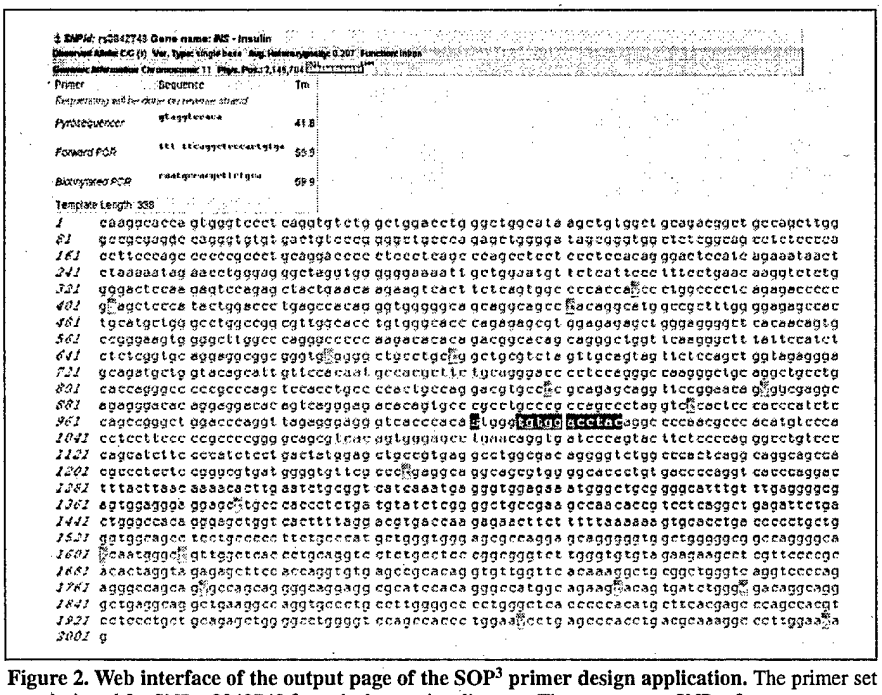


Figure 2. Web interface of the output page of the SOP[3] primer design application. The primer set was designed for SNP rs3842748 from the human insulin gene. The gene name, SNP reference sequence number, allele description, average heterozygosity value, and location of the SNP are indicated in the upper part of the figure. Primers for Pyrosequencing and PCR, along with the calculated length of the PCR amplification product, are given in the central part of the figure. A nucleotide motif at the 5′ end of the forward PCR primer, separated by a space from the rest of the primer sequence, signifies the modification to the primer sequence to avoid self-priming during Pyrosequencing. In the lower area of the figure, the chromosomal DNA sequence surrounding the SNP is illustrated. The polymorphic residues are shaded blue while primers for PCR and Pyrosequencing are shaded yellow and red, respectively.

zygosity value), are then returned to the application to facilitate primer design for PCR and, if successful, Pyrosequencing. This flanking sequence also indicates the locations and polymorphisms of all additional SNPs (nearby SNPs) contained in the database. The identification of sites for PCR primer annealing are based on providing primers that result in a PCR product of at least 60 bp in length while avoiding repetitive sequence elements and the formation of primer dimer pairs. The search begins for unique "n-mers" of user-specified length to make up the 3′ end of the PCR primers, and the search begins 30 bases on either side of the SNP to leave room for placement of a sequencing primer and includes querying a sequence string incorporating the other possible nucleotides at all other SNP locations. Once a unique region is located, the potential primer location is extended along the reference sequence until the specified melting temperature is achieved (14). The primer is tested to determine if it contains a predefined representation of each nucleotide residue, which is accomplished through the user-defined residue occurrence threshold setting. As an additional level of specificity, the user can select to incorporate the AT test during primer design. This provides an additional constraint that will reject a potential oligonucleotide if it does not contain at least one A or T residue within the previously defined unique 3′-end region. The primer is then tested for the presence of a palindrome that would result in secondary structure and thus inhibit PCR amplification. Moreover, the forward PCR primer can be designed with additional unique nucleotide sequence at its 5′ end to avoid the formation of secondary structure in the biotinylated template strand, which, during Pyrosequencing, can lead to competing sequencing signal (6,12,15). Once a pair of candidate primers is chosen, it can be rejected for the potential formation of primer dimers by searching for reverse complementarity. If this occurs, the program continues to choose a new forward primer, and then tests for dimerization with the reverse primer. If failing again, another reverse primer is chosen and tested against the previous two forward primers. This continues in a stepwise manner, each time moving further away from the SNP location, until

Table 1. Inputs, Default Values, and Output for SOP3

Input	Default Value
Search Settings	
Entry box for gene name (locus) or RefSeq Number or chromosomal location	gene name (locus)
PCR Primer Design	
T_m[a]	60°C
Flank length (maximum 1000 each side)	1000 nucleotides
Use repeat masker (yes or no)	no
Use extra 5′ nucleotide	yes
Maximum PCR size	500 nucleotides
Minimum foldover	5 nucleotides
Unique n-mer length	6 nucleotides
AT test (yes or no)	no
Residue occurrence thresholds (minimum and maximum)	minimum 14, maximum 40
Pyrosequencing Primer Design	
T_m[a]	40°C
Primer minimum distance from SNP	1 nucleotide
Maximum distance from SNP	30 nucleotides
Function Type	
Can limit search by specific SNP attributes such as: UTR, splice site, synonymous or nonsynonymous change, coding region, or intron.	all selected
Order Results and Save	
Results can be saved to file (yes or no)	no
Results can be parsed by 1 or 2 limits: (heterozyosity, gene name, rs#, location, chromosome, or function).	heterozygosity, ascending
Ouput Display	
Gene name, rs#, and residue change	
Heterozygosity value, primer sequences	
Nucleotide sequence flanking SNP	
Sites of PCR primer annealing	Highlighted in yellow
Sites of Pyrosequencing primer annealing	Highlighted in red
Location of nearby SNPs	Highlighted in blue
Repeat masked regions	Capitalized, highlighted in gray
Predicted program (yes or no)	No

T_m, melting temperature; SNP, single nucleotide polymorphism; UTR, untranslated region; rs#, reference sequence number.
[a]T_m was calculated using the equation $T_m = 16.6*\log[\text{cation concentration}] + 41*(\text{fraction of GC}) + 81.5$, where the concentration of cation was estimated to be 0.1 M (14).

a suitable primer pair is found within the specified PCR product length.

Algorithm for the Selection of Pyrosequencing Primers

Pyrosequence primer design is initiated once a suitable pair of PCR primers has been identified (Figure 1) and occurs under the same basic parameters. Pyrosequencing primers are required to be within a user-selectable number of residues of the site of the SNP (with the maximum being 30 nucleotides). This 30-base maximum helps to streamline high-throughput assays and reduce reagent use. The AT test and the test for residue occurrence thresholds are not performed on the Pyrosequencing primer, but primer dimers and hairpin formation tests are still applied so as to avoid the formation of secondary structure that may interfere with the annealing of Pyrosequencing primer to the biotinylated template strand. Candidate Pyrosequencing primers are chosen on both the 5′ and 3′ sides of the SNP, and the one closer to the SNP location is selected. This step is incorporated to reduce the sample processing time and cost of testing. Note that if the Pyrosequencing primer is selected 3′ from the SNP, the sequence of the PCR and Pyrosequencing primers printed above the sequence map are given as the reverse complement, as is necessary to correctly biotinylate, PCR amplify, and sequence the appropriate DNA strand during Pyrosequencing. Rules for developing Pyrosequencing primers include the initiation of primer design at a unique user-specified nucleotide sequence motif occurring as close as possible to the site of the SNP and within the PCR-amplified region. As was done for the PCR primers, the unique sequence motif defines the 3′ end of the Pyrosequencing primer, which increases primer specificity during the sequencing reaction. The program requires that Pyrosequence primers have a calculated melting temperature (T_m) of at least 40°C (default value) and lack secondary structure consisting of more than a user-specified number of adjacent nucleotide base pairs (14).

Web Interface

Default settings (summarized in Table 1) are presented for PCR and Pyrosequencing primer melting temperatures, minimum allowed secondary structure, and required length of the unique sequence motif at the 3′ end of the Pyrosequencing primer. The application accepts input for the length of additional sequence harvested from the warehoused genomic databases in order to obtain extended sequences flanking the SNP for use during the design of PCR primers. However, the current version of SOP[3], version 1, does not accept user input of individual sequences because the scope of the application is to aid in the development of primers for projects in which large batches of primer sets must be developed. The output can be parsed by gene name, SNP reference number, heterozygosity value, chromosome, or chromosomal location or function. The application allows one to choose the SNPs for which to develop PCR and Pyrosequencing primers based on the SNP location within each genetic structural element. For example, queries can be defined to return PCR and Pyrosequencing primers for SNPs associated with introns, exons,

Table 2. Selected SNPs Developed for Pyrosequence-Based Typing Assays Using the SOP3 Primer Design Application

RefSeq	Gene Name	Description	Chromosome	Gene Element[a]	Alleles	Heterozygosity
rs913746	*COL4A2*	Collagen, type IV, α 2	13	Intron	AC	0.463
rs2272946	*COL4A5*	Collagen, type IV, α 5	X	Exon (nonsyn)	GT	Not reported
rs3747408	*COL4A5*	Collagen, type IV, α 5	X	Exon (syn)	AG	Not reported
rs2056402	*ITGA2*	Integrin, α 2	5	Intron	AT	0.198
rs3212504	*ITGA2*	Integrin, α 2	5	Intron	CG	0.326
rs27890	*ITGA2*	Integrin, α 2	5	Intron	CT	0.203
rs26679	*ITGA2*	Integrin, α 2	5	Intron	CG	0.5
rs1421941	*ITGA2*	Integrin, α 2	5	Intron	CT	0.395
rs3212594	*ITGA2*	Integrin, α 2	5	Exon (syn)	AG	0.192
rs17468	*ITGB1*	Integrin, β 1	10	UTR	CT	0.413
rs1316757	*ITGB1*	Integrin, β 1	10	Intron	AC	0.418
rs2230396	*ITGB1*	Integrin, β 1	10	Exon (syn)	AC	0.187

SNP, single nucleotide polymorphism.
[a]Gene element symbols are synonymous (syn), nonsynonymous (nonsyn), and untranslated region (UTR).

as well as 5′ and 3′ untranslated regions, among others. SNPs associated with amino acid coding changes can also be specified, based on whether the change is synonymous or nonsynonymous. The entry of queries from which primers are to be designed is accomplished by typing gene names, reference sequence numbers, or chromosomal regions into the text box.

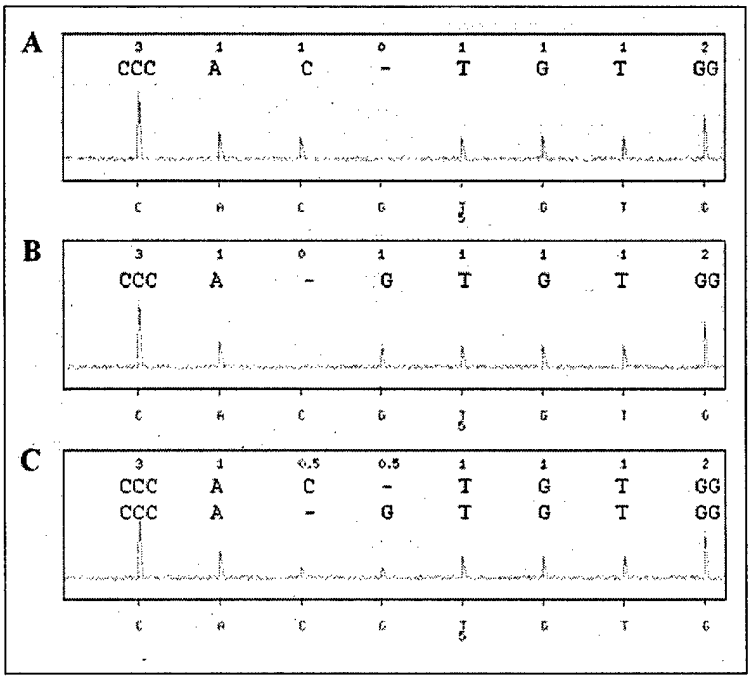


Figure 3. Calculated Pyrosequencing results for human insulin-associated SNP rs3842748. Oligonucleotide primers for PCR and Pyrosequencing are indicated in Figure 2. The SOP[3] application provided a recommended order of nucleotide dispensation events and pyrogram for each (A and B) homozygous and (C) heterozygous genotype. The nucleotide sequence examined is indicated in each panel. SNP, single nucleotide polymorphism.

Figure 2 illustrates an example of the output page of the SOP[3] primer design application. PCR and Pyrosequencing primers were developed for SNP rs3842748, which occurs within an intronic region of insulin. As indicated in the output (Figure 2), the gene name, SNP identifying reference sequence number, and nucleotide polymorphism are provided. The application also returns the dbSNP attributes for average heterozygosity and chromosomal location. Sequences of primers for Pyrosequencing and PCR are indicated along with the expected nucleotide length of the PCR amplification product. In the lower part of Figure 2 is the application's output of the FASTA-formatted sequence flanking the SNP. The predicted pyrograms are also provided with the application output and are shown for SNP rs3842748 (Figure 3) for homozygous (Figure 3, A and B) and heterozygous (Figure 3C) individuals. Pyrograms plot nucleotide dispensation event versus luminescence from the Pyrosequencing reaction on the x- and y-axes, respectively. The predicted pyrograms, as illustrated in the figure, indicate the level of resolution provided by Pyrosequencing for distinguishing various alleles as well as suggesting a potential order of nucleotide dispensation events for each PSBT assay. The SOP[3]-generated nucleotide dispensation order is determined by combining the sequence of fixed and polymorphic residues, as given in the dbSNP database.

Multiple SOP[3]-Generated Primer Sets

The application was used successfully during primer design when tested against a variety of SNPs within loci correlated with risk toward developing diabetes or diabetes-associated complications (Table 2). Reference SNP identifiers, listed in Table 2, were used

as input to query the SOP[3] application. For each SNP that was examined, the application generated a list of candidate primers from which a trio of optimized primers was chosen (primer sequences are given in supplementary Table S1). Using the 12 SNPs listed in Table 2 as an example, the web-based application completed its analysis within 10 s. PCR amplification from genomic DNA was performed using primers developed for 48 SNPs and resulted in 38 (approximately 80%) validated Pyrosequencing assays. Selected examples are illustrated in Table 2. Figure 4 presents the results for SNP rs2056402 located within the gene encoding the α2 subunit of integrin. Pyrosequencing traces from human genomic DNA samples are illustrated for homozygous (Figure 4, A and B) and heterozygous (Figure 4C) individuals selected from our pool of healthy volunteer donors. Of the assays that failed, roughly half were due to poor amplification during PCR, while the rest yielded Pyrosequencing data in which an additional unrelated sequence was present, perhaps due to co-amplification of a pseudogene (data not shown). Table 2 summarizes information from the SOP[3] application's output regarding gene name, reference sequence, genetic element location, allele, and average heterozygosity for a subset of the primers examined. The efficiency of DNA amplification using PCR primers designed by the SOP[3] application are comparable with those reported for web-based software applications developed for designing PCR primers. These applications have reported that 90% or more of the PCR primers tested succeeded in generating PCR amplification products from genomic DNA (16,17). However, SOP[3] is the first software application that can automatically generate PCR and Pyrosequencing primers surrounding one or multiple SNPs in a given genomic region.

DISCUSSION

Pyrosequence-based typing of alleles consisting of SNPs as well as regions of polymorphic DNA has been performed during gene mapping studies to associate disorders in human populations with specific genetic markers (4,6,7,18–21). The methodology is able to score hundreds of alleles daily, which makes it suitable for SNP screening studies involving large numbers of samples. Pyrosequencing provides distinct advantages for genetic typing in that samples can be assayed in 96-well trays, making it compatible with laboratory automation, thus increasing the rate of sample analysis, and the sensitivity of the technology requires little PCR-amplified material for each sequencing reaction (1). PSBT strategies can be designed using a minimal number of nucleotide dispensation events so that an entire 96-well tray can be assayed at a rate of roughly 1 min per base and approximately 10 min for a 10 nucleotide sequence, which is sufficient for the analysis of most SNP and deletion/insertion-containing alleles.

The web-based software application SOP[3] was developed to enhance the use of PSBT for SNP scanning projects in which many SNP assays need to be developed. To that aim, the restrictions applied to PCR and Pyrosequencing primer design have been taken into consideration. For example, PSBT during SNP scanning projects requires PCR primers designed such that the 3′ end of the amplified template does not fold to form a hairpin capable of initiating DNA extension during Pyrosequencing (7,12). Increased Pyrosequencing signal is proportional to the presence of increased concentration of DNA sequencing template in the reaction, a requirement achieved by the design of robust PCR primers. Likewise, Pyrosequence signal is increased by the placement of the Pyrosequencing primer as close to the site of the SNP as possible while obeying the constraints associated with stringent annealing of primer at the Pyrosequencing reaction temperature (1,5). Additional stringency has been achieved by the addition of single-stranded binding protein (SSBP) to the Pyrosequencing reaction as reported previously (7,22).

The current version of the SOP[3] application, version 1, was developed specifically for use in SNP screening projects involving human genomic DNA samples for the association of genetic markers with disease loci. Searching by gene name alone may omit some SNPs with sequence information but without an associated locus name. At this point, only di-allelic SNPs are considered.

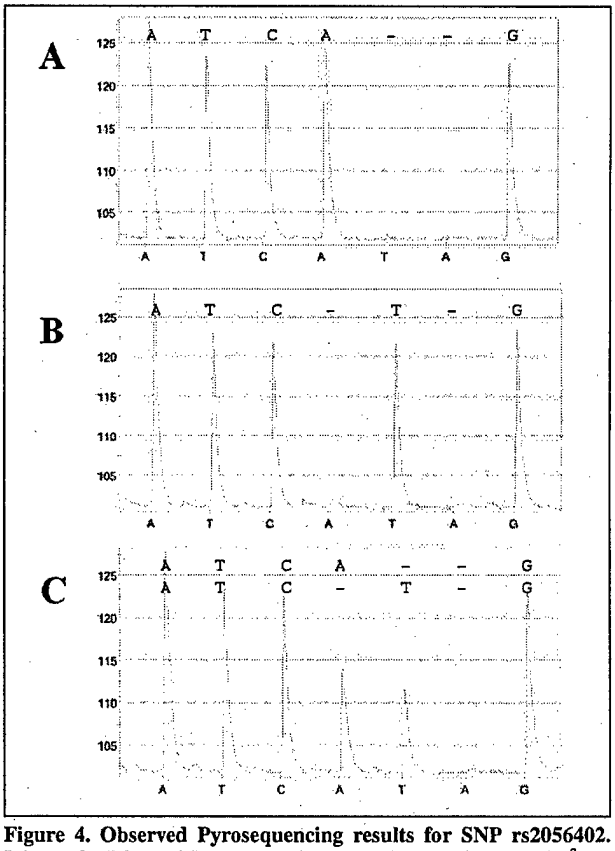


Figure 4. Observed Pyrosequencing results for SNP rs2056402. Primers for PCR and Pyrosequencing were chosen using the SOP[3] application. Genomic DNA was obtained from healthy donor volunteers. Samples genotyped were (A) homozygous AA, (B) homozygous TT, and (C) heterozygous. The nucleotide dispensation order is indicated along the x-axis while the Pyrosequence is illustrated for each sample. Nucleotide disposition A6, following the polymorphic residues, was intended to serve as a negative control for background signal. SNP, single nucleotide polymorphism.

Tri-allelic, or complicated insertion-deletion, are not accurately represented by the current version of the computer program. Primers sets will be designed by the software, however, the third (or fourth) nucleotide possibility of a complicated polymorphism will not be taken into account when searching for unique sequence regions at which to initiate primer selection. The creation of a version of the software for use with the mouse and other model organism genomes is underway and will be added during future updates of the web site. Moreover, the database of SNPs used in version 1 of the application has been downloaded from dbSNP at the NCBI. This database contains values for the average heterozygosity of each SNP as provided in dbSNP. Other databases, such as that at the Hapmap project, also contain lists of SNPs that can be warehoused and developed for use with the SOP^3 application. These can be added in future versions and will allow primer design to focus on validated SNPs from these publicly available sources. The principal advantage of the current version of the SOP^3 application is that it provides a single site for developing primers for PCR and Pyrosequencing, of which previous outputs have been tested and resulted in successful genotyping of human genomic DNA. Additional changes incorporated in future versions of the software application that will improve PSBT of alleles during genome scanning projects may include the development of databases for tracking primer design, which would allow users to report whether primers suggested by the SOP^3 application resulted in useful Pyrosequencing data. Data warehousing of genomic sequences and polymorphisms has increased the efficiency of the design of useful primer sets for PCR and Pyrosequencing by providing a single application for generating these oligonucleotides for laboratory testing.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing interests, commercial or otherwise, regarding the data presented in the manuscript.

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